Biophysical and biological effects from infrared Low-Level-Laser-Therapy

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ABSTRACT OF THE THESIS

INTRODUCTION

Physiotherapists have since the early days of the profession utilized electro physical agents (EPAs) as part of their intervention toolbox. A prerequisite for application of all EPAs is that the applied energy affects biological processes in the body tissue. Low level laser therapy (LLLT) has been used as an intervention for the last three decades to modulate processes in pathological tissue beneath the skin. However, the photoprotective property of the skin is a significant barrier to optical energy applied by LLLT devices. The irradiated electromagnetic energy from LLLT first interacts with the skin where biophysical processes occur. The penetrating part of the energy will then act as an active ingredient in biological processes in subcutaneous tissue.

AIM

The overall aim of this Thesis was to investigate biophysical and biological effects from commercial infrared LLLT devices commonly used in clinical physiotherapy practice.

METHODS

Study I was designed as a blinded placebo-controlled study of repeated measures. The thermal effects from different doses of irradiation from two infrared class 3B lasers was investigated *in situ* in human skin of different skin colours, age and genders. Study II was a basic research study of repeated measures design. The time-profile for energy penetration through skin during 150 sec of irradiation from two infrared class 3B lasers was elucidated *in vivo* in rat skin. Study III was designed as a double-blind randomized controlled trial on effect from a 3 J irradiation dose *in situ/in vivo* in acute rat Achilles tendons trauma.

RESULTS

There was a positive correlation between increasing irradiation doses and increased skin temperature for both lasers in all groups of participants. The skin temperature increased significantly ($p<0.01$) more in dark skin during laser irradiation than in light skin, regardless of irradiated doses and laser type. There were no significant differences in skin temperature between genders or age groups during laser irradiation.

The percentage of energy penetrating through rat skin from the λ=810 nm laser was constant (20%) during 150 seconds of exposure, while skin penetration from the λ=904 nm laser increased almost linearly (38%-58%) and by 50% during the 150 sec of irradiation. The percentage of energy penetrating through rat skin during irradiation was significantly ($p<0.01$) higher from the λ=904 nm laser than from the λ=810 nm laser at all measured time-points.

The biological effect from 3 J half an hour after trauma was significant ($p<0.05$) increased tendon thickness (including the peritendon) in injured Achilles tendons compared to animal's healthy Achilles tendon in the active-LLLT group. These tendon thickness differences were insignificant ($p=0.35$) in the placebo-LLLT group. There were no significant differences in UTS in any group.

CONCLUSIONS

This project has revealed that the biophysical properties of two common types of LLLT devices; λ=810 nm and λ=904 nm, yield significantly different thermal effects in human skin and possess significant different optical penetration abilities in rat skin. These biophysical properties of infrared class 3B lasers are of scientific value as no studies so far have dealt with these effects. These findings support the differentiation made in WALT dosage recommendations for these two types of lasers. The thermal effects in light and medium coloured skin were negligible (<1.5°C) for WALT recommended doses from both laser types, so were the thermal effects in dark skin from the λ=904 nm, 60 mW MOP laser (<2°C). In contrast, the λ=810 nm, 200 mW caused thermal effects (>9°C) above the pain threshold, and indicate that these LLLT devices parameters are probably unsuitable for clinical use in patients with dark skin.

The project also revealed that LLLT irradiation can exacerbate the acute inflammatory process in terms of increased oedema formation. Possible explanations for this somewhat surprising finding include that half an hour may be too early to initiate LLLT after trauma, or that a recently LLLT-treated injured tendon may be more vulnerable than untreated tendons to a repeated injury, or simply that the dose was too high. Future studies are needed to determine these issues.

LIST OF PUBLICATIONS

PAPER I Joensen J, Demmink JH, Johnson MI, Iversen VV, Lopes-Martins RAB and Bjordal JM.

The Thermal Effects of Therapeutic Lasers with 810 and 904 nm Wavelengths on Human Skin

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PAPER II Joensen J, Øvsthus K, Reed RK, Hummelsund S, Iversen VV, Lopes-Martins RAB and Bjordal JM.

Skin Penetration Time-Profiles for Continuous 810 nm and Superpulsed 904 nm Lasers in a Rat Model

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PAPER III Joensen J, Gjerdet NR, Hummelsund S, Iversen VV, Lopes-Martins RAB and Bjordal JM.

An experimental study of low-level laser therapy in rat Achilles tendon injury

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ABBREVIATIONS

ANOVA	Analysis of variance
°C	degrees Celsius
CI	confidence interval
cm	centimetre (10^{-2} meter)
cm^2	square centimetre
ECM	extracellular matrix
EPAs	Electro Physical Agents
g	gram
h	Planck's constant $h = 6.626^{-34}$
Hz	Hertz
J	Joule
kHz	kilo Hertz (10^3 Hertz)
Laser	Light Amplification by Stimulated Emission of Radiation
LED	Light emission diodes
LLLT	Low Level Laser Therapy
MOP	Mean Output Power
mg	milligram (10^{-3} gram)
mK	milli Kelvin (10^{-3} Kelvin)
ml	milliliter (10^{-3} litre)
mm	millimeter
mW	millewatt (10^{-3} Watt)
µm	micrometer (10^{-6})
n	number in a sample
N	Newton
NSAIDs	Non-Steroidal Anti-Inflammatory Drugs
nsec	nanosecond (10^{-9} sec.)
nm	nanometer (10^{-9} meters)
nW	nanowatt (10^{-9} Watt)
p	probability level
PDT	Photodynamic therapy
PGE_2	Prostaglandin-E_2
RCTs	Randomized Controlled Trials
RTUS	Real Time Ultrasonography
sec	second
SD	Standard Deviation
UK	United Kingdom
UTS	Ultimate Tensile Strength
W	Watt
WALT	World Association for Laser Therapy
λ	Wavelength designed by the Greek letter Lambda

CONTENTS

1. INTRODUCTION

1.1 A BRIEF HISTORICAL VIEW ON ELECTRO PHYSICAL AGENTS (EPAS) AND PHYSIOTHERAPY

The first electrotherapy pioneers back in the 19th century were John Wesley and James Graham. They used electrical currents in the treatment of various health disorders from rheumatism and epilepsy to impotence. The first electrotherapy department at a hospital was started by Golding Bird at the Guy's Hospital, London, in 1836 (Selcon, 2001). Investigative reports on effects from physical modalities in musculoskeletal conditions were published as early as in 1931 in Journal of American Medical Association (Wolfson, 1931). The article concluded that *"the result obtained by the combined therapy, is encouraging, in that quicker and often earlier recovery has been attained"*. This sparked an interest in research on physical modalities and several papers were published just before and after World War II. Studies from this early period focused either on effects from electrical stimulation of the peripheral nervous system, or thermal stimuli delivered by hot baths, ultrasound, infrared heat lamps or ice packs (Pohlman et al., 1939; Schaubel, 1946; Gersten et al., 1949). The overriding hypothesis from this period was that heating induced by physical modalities would speed up metabolism and accelerate healing and restoration of damaged tissue (Kohn and Rollerson, 1959). One of the great challenges was to find out how the skin barrier affected the transfer of physical energy to the tissues beneath.

During the 1950's, physiotherapists took an interest in further development of massage and manual techniques. Orthopaedic surgeon James Cyriax at St. Thomas Hospital in London had great influence on the physiotherapy pioneers in manual therapy (Lamb et al., 2003). Friction massage, joint mobilization/manipulation and passive stretching were included in the repertoire of manual modalities. An important perspective in this development of the profession was that physiotherapists extended their scope of practice to include examination of patients. The pioneers of manual therapies developed an extensive examination system and advocated that treatment decisions should be based on specific clinical pathoanatomical tests to reveal which tissues were affected, rather than a less specific diagnosis set by others (Refshauge et al., 1995).

Much of the early physiotherapy research was performed by medical doctors and included aspects of pathological and therapeutic mechanisms on a microscopic or organic level, as well as clinical studies. When Physiotherapy entered the research arena, more of the published studies were of a clinical nature focusing on global treatment effects rather than biological mechanisms of action.

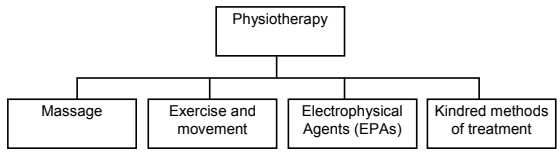
In the field of EPAs it also became clear that much of the literature on physical modalities regarding penetration and absorption of energy in the skin barrier was sparse and quickly became outdated. Another poorly investigated area for physical modalities is related to their time-effect-profile, and consequently the importance of tailoring the timing of interventions to the pathophysiological processes at play.

1.1.1 EPAs IN PHYSIOTHERAPY

Since the early days of physiotherapy, EPAs modalities have been part of our intervention toolbox. An underlying premise of all EPAs interventions is that application of energy can alter the living body's biological processes. The biophysical effects from tissue heating affect the body's homeostasis and metabolic reactions, and induce vasodilatation and specific tissue changes like tendon extensibility and fluids viscosity (Lehmann, 1953; Rivenburgh, 1992; Rennie and Michlovitz, 1996; Collins, 1996; Watson, 2000; Robertson et al., 2006).

In physiotherapy, different interventions are combined to achieve an optimal intervention package (Figure 1). EPAs are often used for preparing the tissue for local manual techniques or therapeutic exercise. EPA modalities are rarely used alone as monotherapies (Watson, 2000; Robertson et al., 2006).

Figure 1. Scope of physiotherapy practice, pillars of treatment



(BMJ, 1929; CSP, 2006)

The role of EPAs over recent decades has changed from being preparative modalities for other interventions, to becoming a more autonomous modality which can modulate pain and physiological processes during the inflammatory, proliferation and remodelling phase of tissue repair (Robertson et al., 2006; Kitchen and Young, 2008). In this perspective EPAs can be interpreted as competitor and substitute for painkiller drugs rather than a supplement to exercise and movement therapies.

Technological development has both improved the number of electrophysical treatment devices, and increased the assortment of electrophysical examination devices suitable for use by physiotherapists. At the World Confederation for Physical Therapy (WCPT), in Amsterdam 2011, where the International Society for Electrophysical Agents in Physical Therapy (ISEAPT) was implemented as an expert subgroup under WCPT, electrophysical diagnostic and evaluation methods were included in the definition of EPAs in physiotherapy (WCPT- ISEAPT, 2011).

1.2 A BRIEF HISTORICAL VIEW OF RESEARCH ON LIGHT

The theory categorizing light as being of electromagnetic nature was proposed by Faraday in the mid-19th century (Tyndall, 1922), and put forward by Maxwell in *"A Dynamical Theory of the Electromagnetic Field"* (Maxwell, 1864). The scientific evidence for the analogy of light and electrical wave motion was demonstrated around the turn of the 19th to 20th century by Lorentz and Zeeman (The Nobel Organizations, 2012a). About the same time, Planck deduced the relationship between energy (E) and frequency (v) of radiation, $E=hv$ (The Nobel Organizations, 2012b). In the early 20th century Millikan made a direct determination of Planck's constant h, as well as the elementary charge of a single electron (Millikan, 1913).

Another important contribution to physics of light came from Einstein. Experiments like Hertz's observations of charged objects losing their charge after light illuminations could not be explained by the wave theory. Einstein came up with a theory of light sometimes behaving as particles - called the photoelectric effect. The photoelectric effect is a phenomenon where particles are emitted from matter after the absorption of energy from electromagnetic radiation (The Nobel Organizations, 2012c).

During experiments of the scattering process, Compton demonstrated that for each scattering electron there are scattering photons (Compton, 1927). The photoelectric effect, together with Compton's scattering effect, have contributed to our understanding of the wave-particle duality of light (Ekspong, 1999). During the 20th century there was much research in physics on elementary particles, and in the 1950s Kastler showed that electrons can be put into excited sub-states by polarized light. Based on "amplification of stimulated emission of radiation" in the specter of light (laser) Basov/Prokhorov and Townes developed the first lasers in the late 1950s (Karlsson, 2000), and Maiman reported in 1960 his creation of the first ruby laser (Maiman, 1960).

The application of lasers range from low power lasers for purposes such as remote controllers and pointers, therapeutic devices for normalizing tissue homeostasis and reducing pain, to high power lasers for medical surgery and metal cutting devices for industrial use.

1.2.1 LOW-LEVEL-LASER-THERAPY (LLLT) LASERS

A laser consists of a lasing media in a resonating cavity, and an energy source. The supplied energy "pumps up" the lasing media to stimulate emission of radiation. Different types of lasing media have their specific wavelengths of radiation. Lasers are characterized by emitting monochromatic and coherent light. The monochromatic light occurs as it is clustered around a single wavelength. By synchronizing the light's "rhythm" it gets a high degree of coherence.

Lasers used for LLLT are typically of class 3B, classified by an average output power range between 5-500 mW (IEC, 2007). The unit for irradiated energy per second is W, also termed the mean output power (MOP). Commonly used wavelengths in LLLT are in the red- and near-infrared band (λ=600–1000 nm) (Karu, 2007), where the energy absorption in water and cutaneous melanin pigment is low (Anderson and Parrish, 1981).

LLLT lasers can operate in different output modes; continuous or pulsed. Continuous mode has a constant output power in the emitted energy, and MOP is equal to pulse power output. In pulse mode, MOP is a product of pulse power (W), pulse width (sec)

and pulse per second (Hz), as it is an energy average of the total amount of energy in pulses per second. There are two types of pulse mode in LLLT: Chopped-pulsed mode where pulse peak power is <500 mW; and superpulsed mode with high pulse peak power far above the 500 mW limit, but of ultra-short duration in the nanoseconds range (10^{-9} sec.) and long pauses making the average output of typically less than 100 mW MOP.

The unit for delivered energy in an irradiated point in LLLT is Joule (J), also called the energy dose. Another element in LLLT is the laser beam spot size (cm^2) or the cross section area. The spot size is critical for power density (mW/cm^2) calculations but is hard to define as the beam power distribution is not homogeneous. In addition to the spot size, the probe lens also shapes the laser beam. Commonly used laser probe lenses are flat or convex. A flat lens does not change the shape of the beam, while a convex lens reduces the natural angle of beam divergence caused by the laser diodes.

1.2.2 LLLT AS THERAPEUTIC INTERVENTION

During the 1960s, experimental research was done with gas lasers in biological tissue (Goldman et al., 1963; Mester et al., 1968a), and the first papers on the biostimulating effects from LLLT irradiation with HeNe gas lasers were published (Carney et al., 1967; Mester et al., 1968b; Goldman et al., 1968).

Most of the early LLLT research focused on possible stimulation of cell proliferation. Various *in vitro* studies have shown that LLLT can stimulate cell metabolism in HeLa cells (Karu et al., 1984), Schwann cells (Van Breugel and Bar, 1993) and fibroblasts (Young et al., 1989; Loevschall and Arenholt-Bindslev, 1994; Yu et al., 1994). Similar *in vivo* animal studies with experimental injuries of various tissue types like skin (el Sayed and Dyson, 1990), cartilage (Nicolopoulos et al., 1996), tendons (Reddy et al., 1998), muscles (Bibikova and Oron, 1993) and nerves (Rochkind, 1992; Anders et al., 1993) found positive effects from LLLT on the healing process. Partly in parallel with this, a few researchers took an interest in investigating possible LLLT effects in inflammation, but only scattered studies were performed with contradictory results (Young et al., 1989; Honmura et al., 1992; Bouma et al., 1996). During the late 1990s LLLT interest picked up again, and Japanese dental research groups were

particularly active in this period (Shimizu et al., 1995; Ozawa et al., 1997; Sakurai et al., 2000; Takema et al., 2000; Nomura et al., 2001).

Publication activity for LLLT research was picking up around the turn of the 21st century. LLLT was demonstrated to act biomodulatory through light absorption of photoreceptors (Karu, 1999). These photoreceptors absorb light leading to a cascade of metabolic reactions in cells (Karu, 2008). It has been demonstrated that cytochrome c oxidase (the terminal enzyme in the electron transport chain) is the photoreceptor and that the initial reactions result in changes in the presence of nitric oxide (NO), reactive oxygen species (ROS) and adenosine triphosphate (Karu et al., 2004; Karu et al., 2005; Karu et al., 2008). Recently, the anti-inflammatory effects from LLLT have been extensively investigated and several aspects of the inflammatory process can be modulated by LLLT. In studies investigating the gene expression profiles of human fibroblasts, most genes enhance cell proliferation and/or suppress apoptosis, directly or indirectly, from LLLT (Yu et al., 1994; Zhang et al., 2003; Frigo et al., 2010; Chen et al., 2011). LLLT also reduces expression of pro-inflammatory mediators (Rizzi et al., 2006; Pires et al., 2011) and regulate oxidative stress (Fillipin et al., 2005; Rizzi et al., 2006; Moriyama et al., 2009).

An increasing amount of fairly homogeneous results from *in vivo* studies strengthened the support for a positive anti-inflammatory response from LLLT in injured tendons (Salate et al., 2005; Elwakil, 2006; Oliveira et al., 2009; Pires et al., 2011; Marcos et al., 2011; Marcos et al., 2012), -muscles (Rizzi et al., 2006; Mesquita-Ferrari et al., 2011; de Souza et al., 2011), -joints (Moriyama et al., 2005; Moriyama et al., 2009; Pallotta et al., 2012) and in soft tissue (Albertini et al., 2004).

A pain reducing effect from LLLT has been demonstrated in clinical studies on human tendinopathies (Stergioulas, 2003; Bjordal et al., 2006b; Stergioulas, 2007; Stergioulas et al., 2008), neck pain (Gur et al., 2004; Chow et al., 2006; Chow et al., 2009; Konstantinovic et al., 2010), myalgia (Gur et al., 2002; Chow et al., 2009), low back pain (Jovicic et al., 2012) and knee osteoarthritis (Gur et al., 2003; Hegedus et al., 2009). The mechanisms behind pain relief are not fully understood. A possible explanation is a nerve blocking effect from LLLT that was demonstrated by Chow et al. (2007) in laboratory study. Also, suggestions of pro-inflammatory cytokines as a trigger for pain (Brenn et al., 2007; Kawasaki et al., 2008), makes the anti-inflammatory effects a possible explanation for pain relief.

1.3 THE SKIN

The skin is the largest organ of the body, and its` outer boundary for protection. The skin is the first line of defense against intrusions and infections but also a barrier for energy applied from EPAs used in physiotherapeutic treatment. Mammalian skin consists of epidermis and dermis. The thickness of both skin layers varies with anatomical location in the body.

Human epidermis thickness varies from 0.04 mm to 0.4 mm, and is thinnest on the trunk and thickest in the palm of the hand and under the sole of the foot (Millington and Wilkinson, 2009). About 5-10% of cells in epidermis are melanocytes (Holbrook et al., 1988). The type and amount of melanin in the epidermis determine our skin colour (Alaluf et al., 2002; Yamaguchi et al., 2007), and play a central role in photo-protection of the skin (Giacomoni, 1995; Taylor, 2002). Dermis thickness ranges from 1 mm to 4 mm (Igarashi et al., 2005). Dermis is rich in blood vessels and sensory nerve endings, these are *inter alia* involved in thermoregulation (Ovalle and Nahirney, 2008). Hemoglobin also has light absorption bands in the red- and near-infrared radiation spectrum (Mobley and Vo-Dinh, 2003). The skin is attached to underlying structures by hypodermis, a subcutaneous fatty layer. The hypodermis also contains blood vessels and nerves (Kanitakis, 2002; Guyton and Hall, 2006).

Human skin appearance, structure, and biophysical properties differ with body location and between individuals according to their race, age and sex (Taylor, 2002; Costin and Hearing, 2007; Millington and Wilkinson, 2009). The skin ageing process leads to loss of collagen and elastin fibers; reductions in the size of epidermal, dermal and subcutaneous layers contribute to fragility, loss of laxity and a dry fine wrinkled appearance of the skin (Montagna and Carlisle, 1979; Leveque et al., 1984; Yaar et al., 2002; Farage et al., 2007). Genetic disposition and hormones also have an influence on the skin structure. After the menopause, female skin thickness decreases due to lower levels of estrogen (Shuster et al., 1975; Zouboulis et al., 2007).

Rat epidermis thickness varies from 0.01 mm to 0.07 mm, and dermis thickness ranges from 0.5 mm to 2.2 mm (Bronaugh et al., 1983; Regan Thomas, 2005), which is not unlike the human skin in miniature.

1.3.1 OPTICAL PROPERTIES OF SKIN

LLLT is the application of light typically of wavelengths in the red and near infrared range (λ=600–1000 nm). Radiated energy may be reflected off the skin surface, absorbed within-, or penetrate through the skin. The reflected part of incident radiation of red and near-infrared wavelengths with a beam angle of 90 degrees to the human skin is small, ranging between 4% and 7% (Anderson and Parrish, 1981). Radiation penetrating the outer skin layer is partly absorbed by melanin in the epidermis and hemoglobin in the dermis (Kollias, 1995; Prahl, 1999; Jacques, 2001). Energy absorption by melanin in the skin decreases with longer wavelengths in the interval of λ=600-1000 nm (Anderson and Parrish, 1981; Mobley and Vo-Dinh, 2003; Bashkatov et al., 2005; Bashkatov et al., 2011; Nedosekin et al., 2012). Absorption by hemoglobin also decreases towards longer wavelengths with negligible absorption at wavelengths above λ=650 nm (Prahl, 1999; Roggan et al., 1999; Barun and Ivanov, 2003; Bashkatov et al., 2005; Bashkatov et al., 2011).
Within the skin, part of the radiated energy is refracted and scattered in random directions. This scattering contributes to extending the distribution of energy beyond the collimated beam. Scattering is strongest from wavelengths under λ=500 nm and decreases with longer wavelengths (Kollias, 1995; Bashkatov et al., 2005; Bashkatov et al., 2011).

During LLLT irradiation the probe is usually held in skin contact. With the laser probe in skin contact, reflected energy, in addition to the probe's diode and electronics, will heat the tip of the probe. This again will affect temperature in the surrounding skin. Therefore, measuring the thermal effect on human skin is an indirect way to investigate whether the energy from LLLT is absorbed by, or if it penetrates through, the skin.

1.3.2 LLLT'S SKIN PENETRATION ABILITIES

In order to interact with deeper tissue the radiated energy from LLLT has to penetrate through the skin barrier. Two decades ago, it was stated that most of the energy from λ=632.8 nm and 820 nm lasers was absorbed within the first 0.4–0.5 mm in human skin (Kolari and Airaksinen, 1993). But more recent studies have shown that the energy from λ=850 nm laser was only reduced by 66% after being transmitted through human skin

flaps of 0.784 mm thickness (Esnouf et al., 2007). Another study in female breast skin flaps demonstrated that 50% of the energy from λ=694 nm lasers actually penetrates at least 1 mm downward through the human skin (Topping et al., 2001).

Biophysically, the ability of radiated energy to penetrate tissue is dependent on the laser's wavelength. Light in the interval of infrared wavelengths (λ=750-1000 nm) penetrates tissue better than light in the interval of red wavelengths (λ=600-750 nm) (Anderson and Parrish, 1981; Stolik et al., 2000). Bashkatov et al. (2005) calculated optical penetration depth (δ=light attenuation to 37%) to be 1.5 mm from red wavelengths (λ=600 nm) and 3.5 mm from infrared wavelengths (λ=1040 nm). In clinical studies laser irradiation in skin flaps has shown an almost linear increase in penetration with wavelengths from λ=450 nm to 1030 nm (Ackermann et al., 2002). A similar wavelength dependent penetration depth was also found in a clinical study where a wavelength λ=675 nm penetrated slightly better than a wavelength of λ=632.8 nm in human skin flaps (Kolarova et al., 1999). Other studies have reported similar findings with wavelength-dependent penetration in animal skin (Beek et al., 1997; Enwemeka, 2001).

1.4 TENDONS

Tendons transmit tensile load from muscles to bone. They consist of an extracellular matrix (ECM) and cells. Even though composites vary from tendon to tendon, the ratio of cells to ECM is approximately 1:4 (Kannus, 2000; Nordin and Frankel, 2001; Kjaer, 2004). Cells have the essential function of maintaining ECM by synthesizing and degradation of its substances. Fibroblasts cells are the main cell type in tendons. The solid substance in ECM is mainly collagen type-I mixed with proteoglycans that acts as a binder stabilizing the collagen structure. This morphological structure gives tendons the unique mechanical ability to tolerate high unidirectional tensile load (Jozsa and Kannus, 1997; Riley et al., 1994; Kjaer, 2004; Rumian et al., 2007).

Literature on tendons often state that tendons have relative limited vascularization and low metabolic rate (Jozsa and Kannus, 1997; Kannus, 2000; Cook et al., 2002; Kjaer, 2004; Theobald et al., 2005; Sharma and Maffulli, 2006), which is projected from less blood vessels in tendinous tissue (Lang, 1960; Lang, 1963) compared to muscles (Jozsa and Kannus, 1997; Kjaer, 2004).

However, some studies have demonstrate that collagen synthesis (^{13}C- and ^{15}N-prolin protein fraction synthetic rate per hour) is higher in human tendons than in muscles (Babraj et al., 2005), and that it has an acute increase following exercise (Miller et al., 2005). It has also been demonstrated that vascularization in peritendinous tissue increases significantly following exercise (Langberg et al., 1998; Boushel et al., 2000), which indirectly can interpret an increased intratendinous blood flow.

As aging progresses, tendon metabolic rate and mechanical properties as tensile strength and stiffness decrease (Kannus, 2000; Nordin and Frankel, 2001).

There are relatively few nerves inside tendons. Nevertheless both the myotendinous and the osseotendinous junction are well innervated, but in long tendons the mid-portion section are usually poorly innervated (Andres et al., 1985; Jozsa and Kannus, 1997).

The morphological structure and chemical composition of tendons are identical in humans and other mammals (Amiel et al., 1984; Nordin and Frankel, 2001).

1.4.1 TENDINOPATHY

The term tendinopathy describes all types of non-rupture tendon disorders. Human tendinopathy is probably a result of multiple factors and with potentially differing aetiology. Research on the aetiology of tendinopathy is complex, and even though tendinopathies have been exhaustively studied through the last decades, the exact etiologic initiation of tendon pain remains unclear (Jozsa et al., 1990; Almekinders and Temple, 1998; Maffulli et al., 2003; Wang et al., 2006; Cook and Khan, 2007).

The origin of human tendinopathy is commonly designated to overuse injuries (Khan and Maffulli, 1998; Almekinders and Temple, 1998; Cook et al., 2002; Maganaris et al., 2004). Overuse is suggested to be critical for development of tendon pain, but mechanical overload is not the sole reason for tendon pathology (Almekinders and Temple, 1998; Cook and Khan, 2007). Human tendinopathy also occur following external trauma to tendons (Wedderkopp et al., 1997; Agel et al., 2007; Garau et al., 2008).

Acute tissue injuries induce a complex cascade of actions in the immune system, including activation of integrin's and cytokines (Kindt et al., 2007). The inflammatory

processes are within days gradually undertaken by tissue repair, and during the next weeks to months tissue remodeling processes will reestablish the basal synthesis /degradation homeosthasis (Koob, 2002; Lin et al., 2004; Sharma and Maffulli, 2006).

Human tendinopathies are often irresponsive to treatment and around one in five patients develop chronic symptoms (Jarvinen et al., 1997; Almekinders, 1998; Cook et al., 2002). It has been suggested that chronic tendinopathy is a consequence of a failed healing response in the immune system (Sharma and Maffulli, 2005a; Longo et al., 2009).

The pathology in tendinopathies, either caused by overuse or trauma, results in an inflammatory response with extravasation and tendon swelling (Kindt et al., 2007). Swelling can occur as both intratendinous and peritendinous edema. These pathoanatomic changes seen in tendinopathies can be traced during the tendon's inflammatory- to remodeling phase, and our experience is that increased tendon thickness in humans can be observed long after the tissue becomes pain-free.

1.4.1.1 ANIMAL MODELS IN TENDINOPATHY RESEARCH

Human tendinopathies are multifactorial, and research in human tendinopathies is complex with ethical limitations in inflicting and evaluation methods. Use of animal models in tendinopathy research has the advantage of incorporating invasive evaluation techniques, detailed tissue examination and analysis of biochemical substances. Animal models may be useful in reproducing some aspects of human tendinopathy by providing researchers with the power to control variability (Warden, 2007; Lui et al., 2011).

In the literature, animal models of tendinopathies can be grouped in two categories: Models where tendinopathy is induced by chemical agents; and models which induce tendinopathy through mechanical load or injury (Lake et al., 2008). The latter is commonly done by excessive repetitive loading and can be appropriate in investigating tendinopathy related to overuse (Carpenter et al., 1998; Soslowsky et al., 2002). Increased levels of inflammatory mediators have been found in animal tendons after excessive activity (Perry et al., 2005). These findings fall in line with studies on healthy human tendons

exposed to excessive activity (Langberg et al., 1999; Langberg et al., 2002; Boesen et al., 2006). Another model for mechanical load is inflicting a blunt tendon trauma transversely over the tendon fibre direction. Experimentally, this can be done by a mini-guillotine where a blunt metal blade inflicts a crush trauma to the tendon. Studies on rat Achilles trauma by such a mini-guillotine reveal significant histological changes compared to healthy tendons. The injured tendons yield a significant difference in collagen fibre alignment (Oliveira et al., 2009) and increased number of vessels (Salate et al., 2005) 3 days after the injury. Another study has found moderate inflammation in injured tendons 24 hours, 7, 14 and 21 days after trauma with this model (Fillipin et al., 2005).

1.4.2 LLLT IN TENDON TREATMENT

LLLT has been used in lots of clinical studies on human tendons. In a review of LLLT administered for lateral elbow tendinopathies (within the WALT therapeutic windows), LLLT seemed to offer pain relief and reduced disability (Bjordal et al., 2008). In an intervention study on Achilles tendinopathies, pain relief experienced by patients was significantly better after 4, 8 and 12 weeks of eccentric exercise and LLLT compared to eccentric exercise and placebo LLLT (Stergioulas et al., 2008). In another study, it was demonstrated that LLLT modulates inflammation by reducing PGE_2 in activated Achilles tendinopathy in human (Bjordal et al., 2006b).

In laboratory studies, LLLT has been performed on a variety of different pathological conditions, including injured animal tendons. Histopathological improvement in the LLLT treated groups has been observed in terms of increased collagen production (Reddy et al., 1998), improved collagen bundles organization (Carrinho et al., 2006; Elwakil, 2006; Arruda et al., 2007; Oliveira et al., 2009), and increased number of small blood vessels (Salate et al., 2005). In most of these laboratory studies the effect from LLLT has been evaluated after 3 to 21 days of treatment.

Lesser research focus has been given to the spontaneous effect from LLLT within 24 hours after injury and acute tendon inflammation. However, after induction of inflammation followed by 3-4 LLLT sessions, tendons have exhibited lower

concentrations of inflammatory markers in the LLLT-treated groups when compared to no treatment controls 24 hours after injection (Aimbire et al., 2006; Correa et al., 2007).

1.5 MEASURING METHODS

In the 1990s a shift in interpretation of research results was seen with the evolution of evidence-based practice. Seeking the best available evidence from quality-assessed research findings in clinical trials became popular among health professionals (Bury, 1998; Herbert et al., 2005). Scientific evidence was incorporated into all aspects of physiotherapy practice, including examination, intervention and evaluation. The evidence for good measuring methods was obtained through studies of their validity, reliability, and ability to discriminate, predict and show sensitivity to change. The evidence for appropriate interventions includes proof of some biological action from the active ingredients and a positive clinical response to the intervention.

The interface of irradiating energy and biological tissue is an aspect which requires reliable methods to measure the effects from LLLT. Two obviously measureable biophysical effects from LLLT are; 1) how much of the irradiated energy can be traced in the skin surface, and 2) how much of the irradiated energy penetrates through the skin. The biophysical measure of increased temperature reflects energy absorption and transformation in the skin during LLLT irradiation. This thermal effect can be measured in the skin surface by thermography. The other biophysical effect from LLLT irradiation can be measured by an optical power meter as the fraction of optical energy that penetrates through the skin.

The biological effects from LLLT irradiation in tissue are usually measured at the microscopic level as histological evaluation (Reddy et al., 1998; Salate et al., 2005; Fillipin et al., 2005; Elwakil, 2006; Carrinho et al., 2006; Arruda et al., 2007; Oliveira et al., 2009). In a couple of studies the biological effects have been evaluated at the macroscopic level as tissue mechanical properties (Elwakil, 2006; Ng and Fung, 2008). Another measuring method of tissue on the macroscopic level is RTUS. RTUS measurements are rarely used in small rodents, but RTUS is commonly used to detect pathology in human tissue.

1.5.1 THERMOGRAPHY

Thermography is an imaging method, which measures thermal energy emitted from an object. Thermographic cameras convert invisible infrared radiation to electronic signals, which is then processed to temperature calculations and visible colour images.

Traditionally, human skin temperature is measured by in-contact sensors like the mercury thermometer and the electronic thermometer. Human skin temperature measured by thermography has small inter-subject variability in repeated measurements (Roy et al., 2006; Howell et al., 2009). Body temperature measured on thermographic images has high reliability compared to rectal temperature measured with mercury thermometer (De Curtis et al., 2008). Thermography also yields high sensitivity in diagnosing morphoea (Martini et al., 2002) and in detecting Raynaud's syndrome (Gold et al., 2004).
An advantage of thermographic camera is that it works as a non-contact device with little risk of contact-induced infections.

1.5.2 OPTICAL POWER METER

An optical power meter consists of a sensor measuring energy in an optical signal, and is connected to a console and display unit. The sensors are photodiodes, which are sensitive to specific ranges of wavelengths and power levels.
An optical power meter can be tuned to certain laser wavelengths, where it can measure the mean optical output (MOP) in the optical signal.

1.5.3 REAL TIME ULTRASONOGRAPHY (RTUS)

The piezoelectric crystal in ultrasound transducers converts electrical impulses to sound waves and vice versa. In ultrasonography, sound waves are transmitted into the body where they are reflected differently depending on the type of tissue. The reflected sound waves are collected by a receiver and processed through software to create sonoanatomic images. Tissue of high density (impedance) is displayed in bright grey-shades, and tissue of low density is displayed in dark grey-shades. This grey-scale imaging is called B-mode (brightness mode), and is commonly used in several areas of

medicine. Ultrasonography, for medical purpose, operates with frequencies from 1 MHz to 20 MHz (Siemens, 2010; GE, 2011). The transducer frequency is important for the image resolution: Higher frequencies give higher resolutions image, but have lower tissue penetration depth; and vice versa for lower frequencies. High frequency transducers (10-20 MHz) offer high resolution and are most appropriate for musculoskeletal imaging. Other features emphasizing ultrasonographic images are Coded Harmonics and CrossXBeam: Coded Harmonics enhances the details in images by brighter and more continuous boundaries, and CrossXBeam is a coplanar imaging technic to reduce noise in images (Cruz-Colon et al., 2006).

Ultrasonography is also a real-time imaging method (RTUS). Technical improvement in RTUS the last decades has made this imaging method valuable in the examination of musculoskeletal disorders, and RTUS has become popular among physiotherapists who seek diagnostic identification of tendinopathies. RTUS has high reliability and good sensitivity in detecting tendon abnormalities (Koski et al., 2006; Sein et al., 2007; Bashford et al., 2008), and RTUS has high accuracy compared to *magnetic resonance imaging* (MRI) (Khan et al., 2003; Kamel et al., 2004; Warden et al., 2007) and surgical findings (Hartgerink et al., 2001; Prickett et al., 2003). An advantage of RTUS compared to MRI and *computed tomography* (CT) is the opportunity of examination of tissues real-time during movements.

1.5.4 ULTIMATE TENSILE STRENGTH (UTS)

Tensile testing is performed as mechanical tests of tissue in material-test-machines, where a controlled load moves at a fixed speed pulling on the specimen. Ultimate tensile strength (UTS) is the maximum load and elongation that a specimen can withstand before the breaking point.

The biomechanical properties of tendons have been tested to determine their UTS. In two animal studies, healthy rat Achilles tendons were dissected and stretched to UTS-values of 42.5 N (SD ±5.5) (Kilkelly et al., 1996) and 48 N (SD ±11.0) (Wieloch et al., 2004).

An important and yet unanswered question in tendon research is whether the appearance of tendinopathies affects tendons tensile strength during different phases and severity grades of the disease.

1.6 THE RESEARCH QUESTION

LLLT interventions are commonly used to interact with pathological tissue beneath the skin. However the massive photo-protective property of the skin is a significant barrier which has to be considered to achieve optimal application of LLLT.
The irradiated energy first interacts with the skin, where some of the energy is reflected and some is absorbed. This gives rise to biophysical energy transformation processes and heat production in the skin. The remaining energy from the irradiation will penetrate through the skin, where it can be an active ingredient in ongoing photobiological processes in deeper tissue.

The research question in this Thesis is: What are the biophysical and biological effects from commercial infrared LLLT lasers commonly used in clinical physiotherapy practice?
The scope of the Thesis is to reveal some of the biophysical effects in skin during LLLT irradiation, and biological effects from LLLT in acute tendon trauma (Figure 1, p.28). In this perspective studies were designed to investigate if biophysical differences occur between the two most common types of commercially available LLLT lasers (infrared class 3B) where specifications for wavelength, irradiation mode and MOP differ. Secondly, the thesis will investigate two possible biological macroscopic effects from one of these infrared LLLT laser on a traumatized tendon.

1.6.1 THE OBJECTIVE FOR STUDY I

In LLLT irradiation some of the optical energy is converted to heat, when it is absorbed in biological tissue. During application of LLLT, the probe is usually held in a fixed position in skin contact. The reflected energy from the skin, in addition to energy from the diode and electronics in the probe, will heat the probe tip. An increased temperature in the laser probe tip will heat the skin by convection. In this

way both absorbed and reflected energy from LLLT irradiation contribute to conversion of radiant energy to thermal energy.

There is a lack of research on whether the thermal effects are at play in treatment with commonly used infrared class 3B lasers. A search with the terms ‘‘thermal effects’’, ‘‘human skin,’’ and ‘‘laser’’ in PubMed, Embase, Cinahl, ISI Web of Science and ScienceDirect (October 2009) yielded no clinical studies performed with class 3B lasers.

The objective of study I is to investigate the biophysical effect from LLLT lasers as heat in human skin.

1.6.2 THE OBJECTIVE FOR STUDY II

The few existing clinical LLLT studies that deal with penetration issues, have largely focused either on optical energy loss (Topping et al., 2001; Esnouf et al., 2007) or penetration depth (Kolarova et al., 1999; Enwemeka, 2001). LLLT intervention is typically administered during irradiation periods lasting from 10-20 sec up to a few minutes, but no studies have yet investigated the time-profiles for skin penetration of optical energy from infrared class 3B lasers.

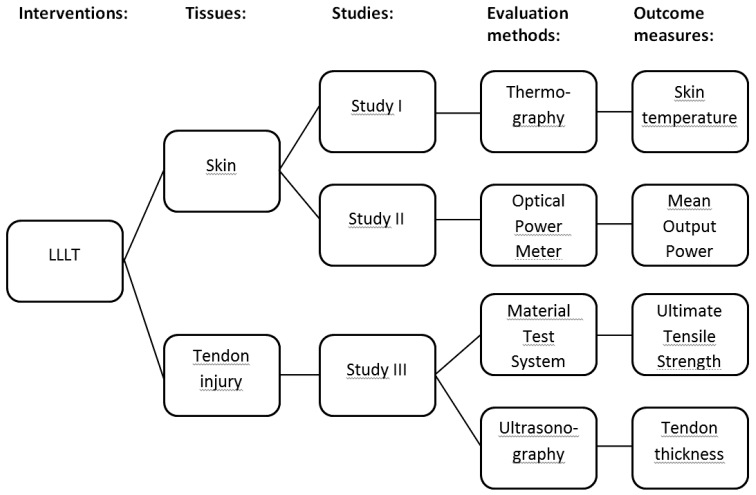
The objective to study II is to determine the biophysical time-profiles for optical energy penetrating skin from LLLT lasers.

1.6.3 THE OBJECTIVE FOR STUDY III

A rat Achilles tendon trauma inflicted in a mini-guillotine model cause histological abnormalities 3 days after an injury (Salate et al., 2005; Oliveira et al., 2009), but it is not known whether these morphological changes affect biological outcomes such as UTS values, tendon structure or edema.

The objective to study III is to investigate whether a LLLT irradiation within half an hour after a tendon trauma cause biological effects in tendon thickness and tendon’s UTS.

Figure 1. Internal relationship between studies in the Thesis.



2. AIM OF THE THESIS

The overall aim of this Thesis is to investigate biophysical and biological effects from commonly used LLLT laser devices where specifications for wavelength, irradiation mode and MOP differ.

The aims of the three studies in the Thesis are:

Study I to investigate the biophysical effects of two therapeutic LLLT lasers on skin temperature in healthy participants of different skin colours, age and genders.

H_0: There are thermal effects in human skin of high doses from infrared LLLT class 3B lasers, varying with irradiated dose and participants' skin colour, age and gender.

H_1: There are no thermal effects from infrared LLLT class 3B lasers in human skin, regardless of irradiated dose and participants' skin colour, age and gender.

Study II to investigate the biophysical rat skin penetration ability of two therapeutic LLLT lasers during 150 seconds of exposure.

H_0: A fraction of the optical energy from infrared LLLT class 3B lasers penetrates through rat skin during commonly used LLLT irradiation times.

H_1: Optical energy from infrared LLLT class 3B lasers does not penetrate through rat skin during commonly used LLLT irradiation times.

Study III to investigate the biological effects of LLLT on tendon and peritendinous edema measured by RTUS and UTS when LLLT is administered half an hour after inflicting an Achilles tendon trauma.

H_0: A single LLLT dose, after an acute tendon trauma, has biological effects on the injured tissue.

H_1: There is no effect from a single LLLT dose after an acute tendon trauma.

3. METHODS

3.1 DESIGNS

Experimental research is characterized by controlled manipulation of variables by the researcher. Controlled manipulation enables researchers to draw causal conclusions about the variable under study. Single-factor experiments are trials where the researcher manipulates only one variable. The single-factor experiment of repeated measures design means that the same subjects are measured under all the levels of the independent variable. The single-factor experiment of post-test-only control-group design, means that post-test is the only basis on which judgements are made about the effect of the independent variable on the dependent variable (Domholdt, 2005).

The investigation of effects from LLLT lasers in biological tissue is best explored in clinical and laboratory single-factor experimental studies. This project includes three trials with these research designs.

STUDY I – is a blinded placebo-controlled study on LLLT in *in situ* human skin. The study has a repeated measures design.

STUDY II – is a basic research study on LLLT in *in vivo* rat skin. The study has a repeated measures design.

STUDY III - is a double blinded randomized controlled trial on LLLT in *in situ/in vivo* rat Achilles tendons. The study has a post-test-only control-group design.

3.2 ETHICS

The protocol for study I was approved by the Regional Committee for Medical and Health Research Ethics (Appl.no: 091.08), and informed consent was obtained from all participants.

The protocol for study II and study III was approved by the Norwegian Animal Research Authority (Appl.no: 20102676).

3.3 SUBJECTS

STUDY I

This study includes healthy volunteers stratified for skin colour, age and gender. Participants were recruited from the staff at Bergen University College, Bergen University and local immigrant associations. The sample consists of 40 persons of both genders, three age groups (<40, 40-60, and >60 years of age), and three skin colour groups (light, medium, and dark skin), (Paper I, Table 1). Individuals with a history of skin disease were excluded.

STUDY II AND STUDY III

These studies were done with Spangue Dawley male rats, weight 250-300 g. Rats were housed four and four in individually ventilated cages under a light cycle of 12+/12-. The humidity was of 55%. The temperature ranged from 20 to 22°C, and the rats received water and food *ad libitum*.

STUDY II - This study includes 62 harvested skin flaps overlaying the rat gastrocnemius muscle.

STUDY III - This study includes 32 Achilles tendons from 16 animals. Animals were divided in two groups of 8 animals (active-LLLT/placebo-LLLT), and housing cages were labeled with group identification.

3.4 INSTRUMENTS

STUDY I

Skin temperature was measured by a thermographic camera (Flir System, ThermaCAM S65HS) and ancillary software (ThermaCAM Researcher Pro 2.8 SR-1). This software includes tools for quantifying the recorded temperature. The camera measures temperatures with a precision of 50 mK at 30°C and has an accuracy of ±2% (Manufacturer´s specification).

Two commercial infrared class 3B lasers were used for irradiation as follows:

i) 810 nm wavelength laser (Thor-DD, UK), operated in continuous mode with 200 mW MOP, spot size 0.0314 cm^2 (Manufacturer's specification);

ii) 904 nm wavelength laser (Irradia, Sweden) operated in superpulsed mode: Peak power 20 W, pulse train frequency 6 kHz and pulses 100 nsec (10^{-9} sec) width (=30.000 pulses per sec), 60 mW MOP, and spot size 0.0364 cm^2 (Manufacturer's specification).
(In Paper I: 60 mW MOP is correct, but pulse specifications are not correct).

STUDY II

The energy penetrating through rat skin was measured with an Optical Power Meter system (Thorlabs Instruments, NJ, USA). The Optical Power Meter system consists of a PM100 display unit with sample rate 6 Hz and an accuracy ±1%, and a S121B Silicon sensor. The S121B sensor input had an aperture with diameter of ~~Ø~~=9.5 mm with an optical power range 500 nW–500 mW at operating temperature 5°- 40°C, and accuracy ±5% (Manufacturer´s specification).

Two commercial infrared class 3B lasers were used for laser irradiation, (the same lasers as described in study I above).

STUDY III

A laser, 904 nm wavelength (Irradia, Sweden), superpulsed mode: Peak power 20 W, pulse train frequency 6 kHz and pulses 100 nsec (10^{-9} sec) width (=30.000 pulses per sec), 60 mW MOP, and spot size 0.38 cm^2 (Manufacturer's specification), with two identical single diode laser probes were used: One with an active laser, and the other an inactive/placebo laser source. Probes were labeled.
(In Paper III: 60 mW MOP is correct, but pulse specifications are not correct).

A servohydraulic testing machine (Material Test System MTS-810, Minnesota, USA) equipped with a calibrated load cell of 500 N and a position transducer of 100 mm, was tuned to a deformation rate of 0.25 mm/sec.

RTUS Logiq-e (GE Healthcare, Minneapolis, USA), operating in B-mode with CrossXBeam resolution and Coded Harmonic imaging, and a 15" display screen. Imaging depth was 2 cm, with three focus areas around the first cm (Paper III, Figure 3). The transducer was a linear array with a frequency of 12 MHz.

The distal tendon grip was set ad modum Forslund, where the angle between calcaneus and Achilles correspond to 30° dorsiflexion of the foot during the UTS test (Forslund and Aspenberg, 2001; Dimmen et al., 2009). The proximal end of the tendon was powdered with 250 µm aluminiumoxid ($AlOO_3$). The proximal tendon grip obtained optimal mortising with a combination of grip and rolling the tendon around the grip.

3.5 PROCEDURES

STUDY I

During pilot work we observed the temperature in both hands on subjects when irradiating one point in one of the hands. The thermography showed heat production strictly located to the irradiated point, and temperature in the irradiated area returned to baseline skin temperature within 1-2 minutes after irradiation. On the basis of this restricted heat localization we decided to use a control area close to the irradiated point. By this we wanted to eliminate effect from systemic temperature regulation mechanisms.

Recommended irradiation doses were selected according to WALT guidelines which are 2 J for 904 nm lasers and up to 6 J for 810 nm lasers (WALT, 2010). With the thermal effects from LLLT irradiation as phenomenon of interest, it was decided also to include doses somewhat above WALT's recommendations. For this purpose, the study was designed with doses from 2 J up to 12 J for both devices.

Laser irradiation was performed at the dorsal side of the proximal phalanx of the index finger. A neighboring area ulnar to the irradiated area in the proximal phalanx of the same index finger was used as a control area (Paper I, Figure 2). The dorsal side of the index finger was chosen as this is a relevant area for LLLT treatment of finger extensor tendons and inter-phalangeal joints.

The laboratory air-conditioning where thermal effects were measured was set to a fixed temperature of 21°C, and this was monitored during the study. This showed a stable temperature throughout the experiment with a room temperature of 21.2°C (±0.2°C).

In order to acclimatize the skin temperature, participants dwelled in the laboratory for 15 minutes before the experiment started. During the experiment participants sat with their hands on a towel placed on a table (Paper I, Figure 1). The distance between the table and the camera was 25 cm. Participants were instructed that during laser irradiation they should report to the investigator if 1) they felt any heat sensation in the irradiated area, and if 2) the heat sensation became so uncomfortable that they wanted the laser irradiation to be stopped.

One investigator operated the thermographic camera, and another investigator administered laser irradiation. Each session lasted 55 minutes per participant with a total of 13 measurements of skin temperature taken. The first measurement was taken before any irradiation (i.e. baseline). This was followed by six measurements during increasing doses of irradiation from the 904 nm laser, followed by six measurements during increasing doses of irradiation from the 810 nm laser. The sequence of the doses was the same for both lasers, and as follows: 2 J, placebo (of same duration time as 2 J), 6 J, 9 J, 12 J and placebo (of same duration time as 12 J). The 810 nm, 200 mW MOP laser deliver 1 J in 5 sec, and the 904 nm 60 mW MOP laser deliver 1 J in $16^2/_3$ sec. Placebo irradiation was delivered using the same laser probe as the active interventions, but the laser was not switched on. Participants were kept unaware of this fact.

During irradiation the laser probe was held stationary in contact with a fixed spot on the skin, and kept in a position approximately 10 degrees from a vertical angle. Between each laser irradiation there was a 3 minute break. Thermographic imaging was used to ensure baseline skin temperature between irradiated doses.

Thermography was recorded during the last 5 sec of each irradiation dose and continued for 1 minute after the end of irradiation (i.e., a total of 1 minute and 5 sec per measurement time-point). The maximum temperatures from the irradiated area and a control area were registered simultaneously by the ThermaCAM (Paper I, Figure 2), and the differences in skin temperature between these two areas constitute the base of the thermal effects.

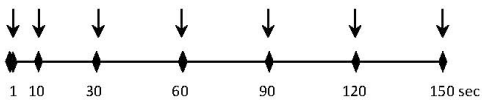
STUDY II

The protocol concerning animal handling is described in detail in the next section – Study III.

LLLT devices typically take from between ten seconds and a couple of minutes to deliver an irradiation dose. Hence, this study was designed with exposure duration of 150 sec.

The Optical Power Meter (PM100/S121B) has five different wavelength options, and it was tuned to the same wavelengths as the respective test-lasers (λ=810 nm and λ=904 nm). Laser's MOP was registered at seven time points; immediately after being activated, after 10 sec, 30 sec and then for every 30 sec until 150 sec (Figure 2).

Figure 2 Measurement time-points



Data were collected in four steps:

Step one – was a measurement of the two lasers MOP directly into the Optical Power Meter. That is with no obstacles between the laser source and the sensor of the Optical Power Meter.

Step two – was a measurement of the two lasers MOP after penetrating a plastic film. That is with a transparent plastic film between the laser source and the sensor of the Optical Power Meter.

Step three – was a measurement of the two lasers MOP after penetrating through a freshly harvested rat skin flap, plus a plastic film. The laser probe was held in skin contact. (The irradiated area on the skin flaps had location corresponding to ~2 cm proximal from the plantar side of calcaneus on the animal).

Step three started with measurement of the immediate laser MOP directly into the Optical Power Meter, and then through a plastic film. After that the skin flap was placed on the plastic film and the sequence of measuring irradiated MOP through skin plus plastic film was performed. This was done for every skin flap and each laser.

Step four – (same procedure as in step three – but the probe was not held in skin contact), the laser probe was now kept approximately 1 mm from the skin surface during the irradiation.

STUDY III

Experimental setup for tendon strength testing - Pilot testing – The deformation rate was discussed with the researchers who carried out a previous study on rat Achilles UTS (Dimmen et al., 2009). Based on this and the experience in testing biological tissue at the current test-laboratory we decided to use a deformation rate of 0.25 mm/sec. The distal grip ad modum Forslund (Forslund et al., 2003) was capable of maintaining a stable grip during UTS testing, but the proximal grip was a challenge. The proximal grip, as described in literature with use of superglue (Chan et al., 2007) and sandpaper (Demir et al., 2004), were not sufficient to avoid the tendon sliding in the grip. Nor did railing the grip surface and rolling the tendon around the grip prevent the tendon from sliding out of the grip. We finally managed to prevent the tendon from sliding by using a combination of swabbing the tendon with alumina powder and rolling it around the grip. With this setup, tendons now ruptured in the mid-portion, a few millimeters from the distal grip.

Simultaneously, the model for inflicting tendon trauma in a mini-guillotine was tested. A trauma was inflicted to the animals' right Achilles tendon. We started with a single trauma, ad modum Lech (Fillipin et al., 2005; Salate et al., 2005; Oliveira et al., 2009), but did not achieve pronounced alterations of UTS on the next day between injured (right) and healthy (left) tendons. We then decided to repeat the trauma on two consecutive days, and now the UTS between injured and healthy tendons became slightly more pronounced, ~10 N. UTS was tested on day 1, 3 and 7 after the last trauma, with small differences in UTS on day 1, 3 and 7. During the half year of pilot tests, the animals included were redundant from other projects at the laboratory, and the groups comprised 2–4 rats.

An irradiation dose of 3 J in this study was based on previous laboratory work in our research group. Preliminary data from an ongoing study with LLLT administered one hour after collagenase injection presented less edema measured at 12 hour after

injection; an irradiation dose of 3 J provided superior results when compared to 1 J and 6 J (now published in Marcos et al., 2011).

The laboratory where the investigations took place had a stable temperature of 24°C (SD ±2°C) and humidity 40% (SD ±10%).

The protocol for animal handling in study II and study III (Figure 3, p.38) was as follow:

Day 1: The animal was anesthetized with isoflurane (Isoba) using an Isotec vaporizer. The hair was shaved from both hind legs. Then animal's right ankle was positioned in full dorsal flexion, and a tendon trauma was inflicted in a mini-guillotine where a 200 g block with an edge 2 mm wide, dropped 20 cm. The trauma was located 9-10 mm from the plantar side of calcaneus, (Paper III, Figure 1). The trauma procedure took 30 minutes to perform. Half an hour later the animal was again anesthetized and subjected to LLLT treatment: One point, 9-10 mm from the plantar side of calcaneus, was irradiated with a dose of 3 J or a placebo dose (time corresponding to 3 J). The animal's tail was marked for each action it went through.

Day 2: The procedures of anesthesia, tendon trauma and LLLT treatment were repeated fifteen hours after the first trauma.

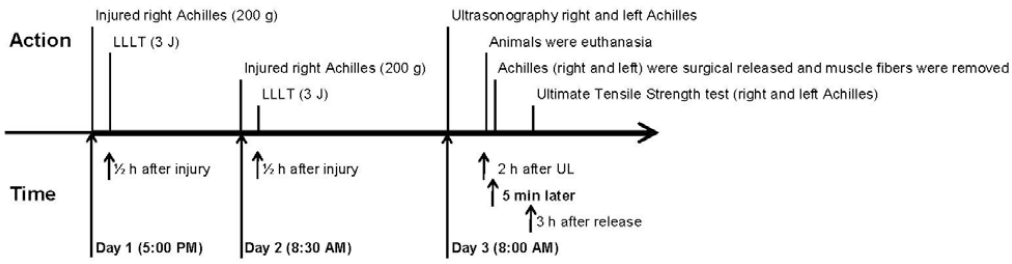
Day 3: Twenty-three hours after the last trauma the animal was anesthetized, and *in situ* tendon thickness (including the peritendon) was measured in the right and left Achilles by RTUS. The Achilles area was screened both in longitudinal and transversal plane; two images were done on each tendon in each plane. Screening in the longitudinal plane was done with the whole length of Achilles in the image (Paper III, Figure 3). When screening the Achilles tendon and the adjacent structures in the transversal plane the probe was moved from the knee in a distal direction until the probe met the Calcaneus, and images were taken in this position.

The animal was again anesthetized two hours later, and given 0.4 ml intraperitoneal anesthetic (Mebumal 50 mg/ml; 1 ml contains 54.9 mg pentobarbital sodium, 400 g propylenglycol, 150 mg strong alcohol and distilled water to 1 ml). The animal was then euthanatized by cardiac arrest (1 ml saturated potassium chloride solution).

The skin overlaying the gastrocnemius muscle from calcaneus to the proximal part of the muscle was dissected free (Paper II, Figure 1). These skin flaps were utilized in study II.

The Achilles tendon was then released with scissors, and the muscle fibers were removed from the tendon with a sharp scalpel. The free tendon was sprayed with water containing 9% sodium chloride, placed in a small plastic bag labeled by a code for group identification, animal number and right/left side, and stored in a refrigerator at 4°C. Three hours later tendons were subjected to UTS testing in MTS-820. Load and deformation data were sampled continuously during this test.

Figure 3 The experiment time schedule



The code for active- and placebo laser probe was not disclosed until the statistical analyses were done. The laser probe output was then measured to determine which probe had been the active one.

3.6 STATISTICS

STUDY I

The differences in skin temperatures between the irradiation spot area and the neighboring control area on the index finger were used in analyses of thermal effects from LLLT irradiation. Analyses were done in subgroups, for each irradiation dose and lasers.

Pearson´s correlation coefficients were calculated to determine relationships between irradiation doses and skin temperature. Student's pairwise t-tests and ANOVA were used for comparison of statistical differences with a significance level set at $p<0.05$.

Microsoft Excel (Microsoft Office Excel 2007) was used for statistical analysis and graphics.

STUDY II

The energy penetrating through skin was calculated as percentage of the amount of energy that penetrated the plastic film measured immediately before skin penetration measurements.

Descriptive statistical analyses on lasers' average MOP, Student's t-test p-value considered significant different, and graphs, at seven time-points, in four conditions, were done by Microsoft Excel (Microsoft Office Excel 2007).

Estimates of linearity in average response between six measured time-points (measurements at 10 seconds were omitted in these calculations) were analyzed by Statistical Package for the Social Sciences (SPSS, ver.19 for Microsoft) repeated measures ANOVA, Generalized Estimating Equations (GEE). The maximum likelihood estimate for linear response between measuring points of 30 sec intervals was calculated with 95% CI.

STUDY III

The difference in tendon thickness (including the peritendon) and UTS, between injured right Achilles treated with active LLLT, - placebo LLLT, and healthy left Achilles, were analyzed by paired t-test in SPSS (ver.18 for Microsoft). Microsoft Excel (Office 2007) was used for graphics.

4. SUMMARY OF RESULTS

STUDY I

The H_0-hypothesis *"There are thermal effects in human skin of high doses from infrared LLLT class 3B lasers, varying with irradiated dose and participants' skin colour, age and gender"* was confirmed, and the H_1-hypothesis *"There are no thermal effects from infrared LLLT class 3B lasers in human skin, regardless of irradiated dose and participants' skin colour, age and gender"* was rejected.

The baseline skin temperature in the proximal part on the index finger after acclimatization was on average 30.7°C (SD ±2.8).

The laser probe tip temperature immediately after irradiation doses from 2 J to 12 J ranged from 30.1°C (SD ±2.2) to 33.7°C (SD ±3.4) for the 904 nm, 60 mW MOP laser, and from 31.8°C (SD ±1.8) to 32.6°C (SD ±2.0) for the 810 nm, 200 mW MOP laser.

After placebo with an irradiation time corresponding to 2 J, the differences in skin temperature between the laser spot area and the control area were -0.9°C (SD ±0.7) for the 904 nm laser, and -0.2°C (SD ±1.2) for the 810 nm laser. After the last dose in the sequence and the placebo with a time corresponding to 12 J, the temperature difference between the two areas was 0.4°C (SD ±1.2) for the 904 nm laser, and 0°C (SD ±1.1) for the 810 nm laser.

All participants completed the six irradiation doses with the 904 nm, 60 mW MOP laser. Participants with light and medium coloured skin completed all six irradiation doses with the 810 nm, 200 mW MOP laser with no reporting of any heat sensation. However, all participants with dark skin reported heat sensations during irradiation from both lasers. Eight of thirteen participants with dark skin withdrew from the experiment with the 810 nm, 200 mW MOP laser because they felt uncomfortable heating in the irradiated area. Two of these participants withdrew during a 6 J irradiation dose (a dose recommended by WALT), four withdrew during a 9 J irradiation dose and two withdrew during a 12 J irradiation dose. Skin temperature at

the time of withdrawal showed a large variation among participants, possibly indicating considerable differences in heat pain tolerance (Paper I, Table 3).

There was a strong positive correlation between increasing irradiation doses and increased skin temperature for both lasers in all sub-groups (Pearson´s r = 0.98–0.99 when withdrawals were omitted from the analysis).

The increase in skin temperature was significantly ($p<0.01$) higher in dark skin compared to light skin for all irradiated doses, regardless of laser type (Paper I, Figure 3 and Figure 4).

Identical doses of laser irradiation resulted in significantly ($p\leq0.001$) higher skin temperatures in dark skin compared to light and medium coloured skin the 810 nm, 200 mW MOP laser (Paper I, Figure 4).
Irradiation doses of 2 J and 6 J with the 810 nm, 200 mW MOP laser induced a threefold increase in temperature of dark skin when compared to the 904 nm, 60 mW MOP laser (Paper I, Figure 3 and Figure 4).

There was no significant thermal effect ($p\geq0.16$ for any comparisons) when irradiating with doses recommended in WALT guidelines (i.e. 2 J for 904 nm, and 6 J for 810 nm), with temperatures increasing less than 1.5°C in light and medium coloured skin regardless of laser type. However, in dark skin there was a highly significant increase ($p<0.0001$) in skin temperature of 6.1°C (SD ±3.2) from a 2 J dose, and 9.2°C (SD ±4.5) from 6 J, with the 810 nm, 200 mW MOP laser. Irradiation with a 2 J dose using the 904 nm, 60 mW MOP laser also induced a significant ($p=0.043$) but non-painful increase in skin temperature of 1.9°C (SD ±1.8) in dark skin.

When equivalent irradiation doses of 6 J or more were administered to light and medium coloured skin there were larger increases in skin temperature for the 904 nm, 60 mW MOP laser when compared with the 810 nm, 200 mW MOP laser ($p<0.05$) (Paper I, Table 4).

There were no significant differences in skin temperature between males and females during laser irradiation in the dose interval of 2-12 J (no p-value lower than $p\geq0.24$).

Adjusting for skin colour by removing data for dark skin from the analyses did not affect this result (Paper I, Figure 5 and Figure 6).

There was a tendency toward higher skin temperatures in the irradiated area in younger participants (under 40 year of age) for both lasers, although comparisons with older participants just barely failed to reach statistical significance ($p \geq 0.06$). There was tentative evidence that there were larger temperature differences between groups with higher doses for the 810 nm, 200 mW MOP laser than with doses recommended by WALT, and that the differences persisted when dark skin data was removed from the analysis (Paper I, Figure 7 and Figure 8).

STUDY II

The H_0-hypothesis *"A fraction of the optical energy from infrared LLLT class 3B lasers penetrates through rat skin during commonly used irradiation times"* was confirmed, and the H_1-hypothesis *"Optical energy from infrared LLLT class 3B lasers does not penetrate through rat skin during commonly used LLLT irradiation times"* was rejected.

Step one (no obstacle, through air only): The MOP from both lasers were stable during the selected 150 sec of exposure. The 904 nm laser increased MOP by 0.3 mW (SEM ±0.4), while the 810 nm laser MOP dropped by 9.8 mW (SEM ±1.9) by the end of 150 sec of exposure time (Paper II, Table 1).
The statistic GEE estimates a slope of 0.25 mW per 30 sec (95% CI: 0.07 to 0.42, $p<0.01$) for the 904 nm laser; and a slope of -1.14 mW per 30 seconds (95% CI: -1.44 to -0.84, $p<0.01$) for the 810 nm laser.

Step two (through plastic film): The plastic film reduced MOP during 150 sec of exposure by 3.6%, or 2.3 mW (SEM ±1.2), on average from the 904 nm laser; and by 6.6%, or 12.7 mW (SEM ±1.0), on average from the 810 nm laser (Paper II, Table 1).
The statistic GEE estimates had the same trend as without any obstacle, but with a less steep slope, 0.16 mW per 30 sec (95% CI: -0.10 to 0.42, $p=0.23$) for the 904 nm laser; and -1.00 mW per 30 sec (95% CI: -1.49 to -0.52, $p<0.01$) for the 810 nm laser.

Step three (through skin - probe in skin contact): Skin flaps from rat hind legs had thickness of 1.2 mm (SD ±0.2) (n=21).

There was no difference between penetration through skin flaps from the left or the right hind legs (t-test values for the two lasers at seven time-points varies from p=0.63 to p=0.91).

The two lasers had statistically different skin penetration abilities. The percentage of energy penetrating through skin from the 810 nm laser, was stable at approximately 20% of the MOP (ranging from 19.5% (SEM ±0.6) to 20.4% (SEM ±0.6) between seven measured time-points) during 150 seconds of exposure (Paper II, Table 1). The statistic GEE estimated slope for the 810 nm laser was -0.26% of MOP per 30 seconds (95% CI: -0.35 to -0.17, $p<0.01$). The percentage of energy penetrating skin from the 904 nm laser increased almost linearly during 150 sec of exposure, from 38.7% of MOP (SEM ±1.4) to 58% of MOP (SEM ±3.5) (Paper II, Table 1 and Figure 2). The statistic GEE estimated slope was 2.29% per 30 sec (95% CI: 1.69 to 2.88, $p<0.01$) for the 904 nm laser.

The two lasers had significant ($p<0.01$) different percentage of MOP penetrating through skin at all measured time-points (Paper II, Table 1 and Figure 2).

Step four (through skin - probe not in skin contact): Both lasers showed the same trends of energy penetrating through skin with the probe not in skin contact as the case was with the laser probes in skin contact. There were significant (t-test, $p<0.01$) differences in the percentage of MOP penetrating through skin with the probe not in skin contact from the two lasers at all measured time-points (Paper II, Table 1 and Figure 2). Even though, the slope of increased penetration during processing time from the 904 nm laser was smaller than with the probe in skin contact: The statistic GEE estimates a slope of 1.42% of MOP per 30 sec (95% CI: 0.82 to 2.03, $p<0.01$) with the probe not in skin contact. In percentages the amount of energy penetrating skin increased from 36.8% (SEM ±2.4) to 44.4% (SEM ±3.0) during exposure with the 904 nm laser (Paper II, Table 1 and Figure 2).

The amount of energy penetrating through skin for the 810 nm laser was slightly higher during irradiation with the probe not in skin contact than with the probe in skin contact: The percentage of MOP was approximately 24% (ranging 23.1% (SEM ±1.7)

to 25.5% (SEM ±2.2)) during the exposure period with the probe not in skin contact (Paper II, Table 1 and Figure 2). The statistic GEE estimated slope was -0.42% of MOP per 30 sec (95% CI: -1.27 to 0.43, p=0.33).

Irradiation with the laser probe in skin contact versus not in skin contact resulted in changes in skin penetration ability for both lasers. These changes are most likely not attributed to the optical laser parameters, but to the different physical shape of the two probe tips. The 904 nm laser probe has a convex protruding lens, which squeezes the skin when the probe is in skin contact. This will cause better penetration with full skin contact than with no skin contact. On the contrary the 810 nm laser probe has a flat folded up lens. Here the metallic ring surrounding the lens will gather and push skin underneath the lens, which leads to less penetration with the probe in skin contact than not in skin contact (Paper II, Figure 3).

STUDY III

The H_0-hypothesis *"A single LLLT dose, after an acute tendon trauma, has biological effects on the injured tissue"* was confirmed, and the alternative hypothesis *"There is no effect from a single LLLT dose after an acute tendon trauma"* was rejected.

Achilles tendon thickness (including the peritendon) measurements in the longitudinal plane by RTUS revealed a statistically significant ($p<0.05$) difference between the injured right Achilles and the healthy left Achilles, in the active-LLLT group. In the group treated with placebo-LLLT, tendon thickness (including the peritendon) side differences were insignificant (p=0.35). The mean Achilles thickness (including the peritendon) on the injured right tendon was 0.93 mm (SD ±0.03) in the active-LLLT group, and 0.73 mm (SD ±0.07) in the placebo-LLLT group. The healthy left Achilles had a mean tendon thickness (including the peritendon) of 0.69 mm (SD ±0.07) and 0.70 mm (SD ±0.10) respectively.

The difference between injured right-Achilles and healthy left-Achilles on transversal plane RTUS images was significant at ($p<0.05$) in the active-LLLT group. In the placebo-LLLT group transversal tendon thickness side difference was insignificant (p=0.16). The mean transversal thickness of the injured right Achilles was 0.70 mm (SD ±0.10) in the active-LLLT group, and 0.63 mm (SD ±0.14) in the placebo-LLLT

group. The mean tendon thickness of the healthy left Achilles in the two groups was 0.51 mm (SD ±0.07) and 0.57 mm (SD ±0.07) respectively.

There were no significant differences in UTS between the injured right Achilles and healthy left Achilles in the active-LLLT group and in the placebo-LLLT group. The mean UTS in the injured right Achilles was 51.11 N (SD ±9.77) in the active-LLLT group, and 57.64 N (SD ±7.80) in the placebo-LLLT group. The healthy left Achilles had an UTS of 53.94 N (SD ±9.80 N) in the active-LLLT group and 59.66 N (SD ±11.86 N) in the placebo-LLLT group.

Two months later we wanted to ensure reliability and measured tendon thickness again on the stored longitudinal images. This revealed an intrarater reliability by ICC(1,1) ranging from 0.68 to 0.83 (unpublished data), indicating moderate reliability.

5. DISCUSSION

This chapter contains sections with general discussion, methodical discussion, further research with pertinent follow-up studies, and finally conclusions.

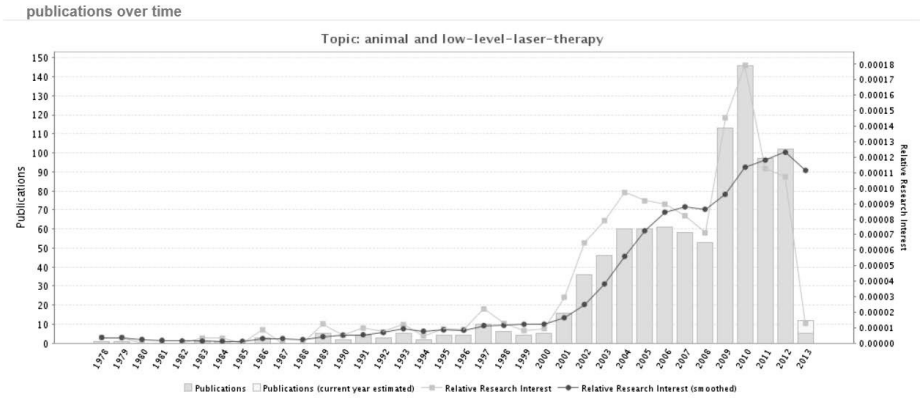
5.1 GENERAL DISCUSSION

This general discussion will focus on the thesis in relation to perspectives of the current project and the physiotherapy research literature, biophysical effects from LLLT in skin, LLLT doses and initiating LLLT after acute trauma, and animal models in tendinopathy.

5.1.1 PHYSIOTHERAPY RESEARCH

Society is constantly changing and this requires health professionals' to adapt to changing demands in the society. Physiotherapists are an important resource available to society's health professionals. They shall always provide the most suitable examination and treatment methods which are best for helping the patients. To meet this requirement research in physiotherapy has increased in recent decades, with more emphasis on evidence-based practice and clinical research (Coronado et al., 2011). The number of RCTs and systematic reviews in physiotherapy published in the Physiotherapy Evidence Database (PEDro) increased almost exponential during the period from 1960 to 2009 (Moseley et al., 2002), and constitutes now more than 23 thousand papers (PEDro, 2013). In line with this increase the quality of RCT of physiotherapy interventions has improved. Since 1960, study's quality score (PEDro score 0-10, where10 is best score on trials internal and external validity) has increased by on average 0.6 points per decade (Moseley et al., 2011). Similar efforts on improved evidence-based knowledge have also taken place in research on LLLT. There has been a sharp increase in the number of animal studies on LLLT since the turn of the twenty-first century (Figure 4). A search in PubMed on the terms "animal" and "low level laser therapy" resulted in almost a thousand papers.

Figure 4 Publications over time on “animal and low-level-laser-therapy”, (search in GoPubMed.com, April 2013)



(GoPubMed, 2013)

In clinical practice physiotherapists use electrophysical devices both in examination of patients and as part of a combined physiotherapy treatment package. Physiotherapists are capable of performing clinical examinations to guide clinical reasoning for optimal timing and optimal combination of treatment modalities. But physiotherapists are not necessarily in-depth specialists in the bordering sciences of physics, physiology or radiology. Still, physiotherapists must sometimes perform scientific research in bordering disciplines when it is necessary to elucidate what mechanisms are at play that causes effects from the various EPAs used by the physiotherapy profession. This kind of translational research has been sparse in physiotherapy science, partly because of physiotherapists´ limited competence in basic science research. The solution to this is of course to initiate collaboration with other researchers in medical and basic sciences. In this perspective, the project at hand is an example of translational research involving a number of disciplines other than physiotherapy.

This project has involved acquisition of new skills that were needed for the realization of the research project. I have acquired skills in imaging techniques such as ultrasonography, thermography and gained qualification for performing laboratory animal studies (Federation of European Laboratory Animal Science Associations, FELASA C-course). An additional benefit from the research project is the confirmation that physiotherapists may be able to acquire these additional skills and to handle them professionally with adequate reliability.

5.1.2 LLLT'S BIOPHYSICAL EFFECTS IN SKIN

The various EPAs used in physiotherapy interventions are designed to deliver different forms of energy to the human body in a therapeutic purpose. Therapeutic lasers deliver light energy to the human skin in order to trigger phototherapeutic reactions in underlying tissues. With the first clinical LLLT studies being published in the 1980s critics soon claimed that the doses used were too low to evoke any noticeable thermal or biological reactions in the human body (Devor, 1990). In the nineties knowledge improved about the biophysical properties of LLLT irradiation. More studies into basic mechanisms led to a different understanding of LLLT effects, which shifted the prevailing perspective to attribute possible LLLT effects to non-thermal mechanisms (Basford, 1995). This perspective may have been challenged by the recent technological developments. New and more powerful class 3B laser devices have been brought to the market by laser manufacturers claiming that "more is better". Theoretically this has re-opened the possibility that thermal effects may be present during LLLT irradiation.

The thermal effects in human skin from infrared class 3B lasers, observed in study I, indicate that laser MOP is an aspect to be considered in clinical settings. The skin temperature increases in a near-linear manner with LLLT irradiation dosage. This dose-temperature relation seems to apply for all combinations of lasers and participant subgroups (age groups, gender and skin colour). The results show that the biophysical effect, heat production, in human skin during irradiation from infrared lasers is strongly related to skin colour. Skin temperatures were significantly higher at all irradiation doses for participants with dark skin compared to other skin colour groups, regardless of laser type. This indirectly indicates a differential influence of light energy absorption as lesser energy seems to be absorbed in light and medium coloured skin than in dark skin. Similarly skin colour dependent absorptions has previously been found in studies with ultraviolet irradiation, where darker skin was found to act as a better photon absorber and photo protection barrier than lighter skin (Giacomoni, 1995; Taylor, 2002; Yamaguchi et al., 2007).

One could be tempted to assume that the observed thermal effect from LLLT solely was a result of irradiated power density (mW/cm^2), but this was not the case. The 810

nm laser with more than three times as high power density as the 904 nm laser induced less thermal effect in light and medium coloured skin.
Although both lasers evoked similarly shaped thermal responses for irradiation doses (J) in the graphs (Paper I), there were other factors that differed. The irradiation time with the 904 nm was more than three times longer as it was with the 810 nm laser for equal doses to be delivered. The longer exposure time component might have caused slightly higher skin temperatures from equal doses in light and medium coloured skin from the superpulsed 904 nm laser than from the continuous 810 nm laser.

Aging of the human skin-structure changes during the lifespan and could possibly influence the biophysical penetration properties for infrared class 3B lasers. Even though there was a tendency to more heat production in younger skin (under 40 year of age) than in older skin (over 60 year of age), these differences in skin temperatures were small and not significantly different between the three age groups included in study I. Although, a type-II error caused by few participants in the oldest age group cannot be ruled out.
Another possible factor influencing biophysical penetration could be gender. A gender difference in sun-sensitivity has previously been reported in the literature, with female skin being more sun-sensitive than male skin (Guinot et al., 2005). However, these gender differences seem to be of lesser importance, as we found no significant differences in the biophysical heating effects in human skin, between genders during irradiation from the two infrared class 3B lasers in study I.

Laser irradiation with doses recommended by WALT resulted in small and therapeutically irrelevant temperature changes (<1.5°C) in light and medium coloured skin. But the observed temperatures in dark skin with doses at, or above, 2 J for the 810 nm laser exceeded the threshold for painful thermal stimuli (Defrin et al., 2002). The significant heating of dark skin caused by the 810 nm, 200 mW MOP laser has implications for irradiation safety, and indicates that lasers with MOP in the 2-300 mW range should probably not be used clinically with doses at and above 6 J in persons with dark skin.

A previous penetration study using LED with an infrared wavelength (λ=840 nm) found decreased light transmission in skinfolds in participants with dark skin, but no significant differences between groups of different skin colours (Nussbaum et al., 2007). However, the power density for the LED source was very low compared to our study and used only 1-2% of the power density used in our study I. Additionally, both ours and their study only included respectively 13 and 15 participants with the darkest skin colour in the sample, and consequently type-II errors cannot be ruled out.

The biophysical ability of irradiation from infrared class 3B lasers to penetrate through skin was larger than expected from previous studies, as between twenty and fifty percent of the irradiated energy penetrated. The biophysical effect time-profile was expected to show a constant amount of energy penetrating through the skin with commonly used irradiation times for both lasers. Small differences between the 810 nm and 904 nm lasers were expected as longer wavelengths have been shown to penetrate slightly better (Ackermann et al., 2002; Bashkatov et al., 2005). However, only the continuous 810 nm laser showed a constant amount of penetrating light over time. As expected, we initially observed that a greater amount of energy penetrated through rat skin from the 904 nm laser than from the 810 nm laser. But the observed 50% increase of penetrating energy during the irradiation period from the superpulsed 904 nm laser was unexpected and can probably not be explained by wavelength differences alone. A biophysical effect time-profile of gradually increasing skin penetration during exposure has not previously been reported in the LLLT literature.

Increased skin penetration during exposure due to photobleaching is a phenomenon in the PDT literature. Photobleaching is the photochemical destruction of fluorescent molecules which consequently reduces the auto-fluorescence of the tissue and thereby causes lesser absorption of light. PDT utilize a photosensitizer in combination with light and tissue oxygenation in cancer treatment (Dougherty et al., 1998), and the photobleaching effect in skin is derived from the photosensitizer (Georgakoudi et al., 1997; Zhu and Finlay, 2008). This makes it inexpedient to extrapolate photobleaching results from the PDT literature to our findings. Although, reduced fluorescence in skin during laser irradiation was found in a recently published study on improving skin Raman spectral quality. Wang et al. (2012) found a fluorescence decrease in human skin of

approximately 25% during 200 sec of exposure from a 785 nm laser with power density 0.29 W/cm^2. This percentage is somewhat lower than our findings from the 904 nm laser. The effect from irradiation modes, continuous versus superpulsed (peak power 12.5 W) was compared in a study on enhanced melanin fluorescence (Kerimo et al., 2011). Irradiation from a 930 nm laser with approximately the same MOP in continuous mode and superpulsed mode showed only small differences in activation of enhanced emission, which indicates that irradiation mode play a minor role in enhanced melanin fluorescence. As discussed, it cannot be said with certainty to what extent this photobleaching observed from the superpulsed 904 nm laser in study II occurred due to irradiation of this particular wavelength and/or the combination of pulse parameters (peak power, width and frequency) compounded in this superpulsed laser or other factors.

The few published LLLT penetration studies either use mathematical modelling or register outcome only for a single time-point. These models make the inherent assumption that penetration is constant and independent of time. In our study II we decided to investigate not only penetration at a single time-point, but to characterize the time-profile for LLLT skin penetration. In this novel area of research, we found it prudent to start by using an *in vivo* model. However, we recognized that further *in situ* experiments are needed in order to verify the presence of the observed penetration variation with superpulsed lasers in clinical settings.

It would be of greater clinical value to pursue the question of skin penetration further by measurements in an *in situ* experiment. This would require a more sophisticated technical procedure with smaller and more advanced optical sensors placed subcutaneously in a surgical procedure in the living subjects. Due to the inherent scattering and backscattering of laser light when passing the skin barrier and different layers of tissue, the optical sensors would also need to be circularly shaped to detect photons from all directions and connected by a thin optical fibre. A natural extension of study II would be to initiate a new *in situ* experiment if possible with suitable sensors, and test if acceptable reliability can be achieved. Finally, with positive results in the *in situ* experiment one could extend this research further to an *in situ* human

experiment with a sensor surgically placed in, for instance, the peritendinous tissue of the Achilles tendon in volunteers, if this would be acceptable to the regional ethical committee. Whereas study II only gives us an indication of the possible relative penetration of LLLT in rats, the above delineated studies if feasible would have shed more precise information on LLLT penetration in human clinical settings.

5.1.3 LLLT DOSES AND INITIATING LLLT

Understanding the LLLT parameters is a challenging exercise for clinicians. Therapeutic lasers are characterized by a complexity of parameters comprising wavelength, output power, spot size and irradiation mode. Additionally, irradiation time seems to play a role, as longer exposure time seems to be more effective than short exposure time (Castano et al., 2007). The optical energy in the laser beam is gradually being absorbed by the tissue it penetrates. Biological tissue can only utilize a certain amount of optical energy, inasmuch as there are no known depositaries for optical energy in biological tissue. This means that irradiation time may have a direct influence on the effectiveness of LLLT irradiation in the biophysical processes taking place in tissue.

There is strong clinical evidence reflected in the WALT dosage guidelines that superpulsed λ=904 nm lasers have lower optimal dose ranges in musculoskeletal disorders than continuous λ=810 nm lasers (Tumilty et al., 2010; Jang and Lee, 2012). It has been proposed that less energy is lost in the skin barrier with the superpulsed 904 nm laser than with the continuous 810 nm laser (Bjordal et al., 2003b). The superpulsed 904 nm laser has both a wavelength-dependent initial larger proportion of energy penetrating through the skin barrier, and increased penetration during irradiation. Calculations on the basis of our findings in study II show that both a 4 J dose from the 810 nm laser and a 2 J dose from the 904 nm laser bring a similar energy dose of 0.8 J through the skin barrier. Consequently, our results from study II support the differentiation between dosages from λ=904 nm and λ=810 nm lasers in WALT dosage recommendations.

In study III rat Achilles tendons were irradiated by a dose of 3 J after injury. An irradiation dose of 3 J is commonly used in clinical practice for human inflammatory conditions and has been adapted from the dose recommendations from WALT (WALT, 2010). It is unclear if LLLT parameters which are effective in humans also can be extrapolated to small rodents. In retrospect, it seems that effective doses in animal studies are somewhat smaller than in humans (Bjordal et al., 2006a), and we may have gone too high in dosage for rat Achilles tendon pathology.

Recent studies with LLLT (λ=810 nm) and doses of 1 J and 3 J following a collagenase-induced tendinitis in rat Achilles evaluated biophysical and biomechanical aspects of the tendons. They found a single dose of 3 J to be most effective for reducing edema significantly 12 hours after injection (Marcos et al., 2011), while the 1 J dose had better UTS values in tendons 7 days after injury (Marcos et al., 2012).

In another recent study with LLLT (λ=904 nm), doses of 0.1, 0.3, 1.0 and 3.0 J were used to see if prevention of skeletal muscle fatigue and muscle damage in rats could be achieved. All irradiated groups except the 3.0 J group showed significantly lower post-exercise creatine kinase activity than the control group immediately after the electrical muscle contraction tests (Leal Junior et al., 2010). Thus, optimal irradiation doses from 904 nm lasers in rat muscles seem to be closer to 1 J, and we may have over-dosed with 3 J in our study III. This could explain the non-significant differences, but possibly not the increased edema observed in the active-LLLT group.

A more likely explanation of the increased edema in the active-LLLT group could be the early initiation of LLLT after injury. Timing the initiating of LLLT after an injury has not been subject to extensive debates in the literature. But in animal studies measuring edema after inducing an injury, LLLT is typically administrated an hour or later (Albertini et al., 2004; Albertini et al., 2007; Bortone et al., 2008; Marcos et al., 2011). It is also conceivable that LLLT treated tendons (on day one of the experiment) might be more vulnerable than untreated tendons to a trauma on the next day.

Hence, a possible negative effect on edema development in inflamed tissue may be restricted to the first half hour after the onset of inflammation (MacKenna et al., 1998; Kyaw et al., 2001; Arnoczky et al., 2002; Werle et al., 2002). In this perspective, our findings may have

important clinical implications as it has been an open question when the earliest time-point for effective LLLT of soft-tissue injury might be.

It is interesting to note that increased tendon thickness is exhibited irrespective of the state of the tendinopathy being in an acute or a chronic phase. In the acute stage of tendinopathy edema may be present in the paratenon or tenosynovia, or intratendinously with loosening cross-links, but without major defects in the longitudinal tendon morphological structure. Increased tendon thickness may also be related to the formation of intratendinous edema after overload or trauma in a partly degenerated tendon (Kongsgaard et al., 2005). After the initial edema partial ruptures of the longitudinal fibre bundles will typically heal with tendon hypertrophy caused by the increased collagen fibre formation during the proliferative phase of tendon repair (Gerriets et al., 1993; Khan et al., 1999; Sharma and Maffulli, 2005b; Fillipin et al., 2005). In this perspective, increased tendon thickness may be present in the early phases due to edema, while tendon hypertrophy may be the cause of increased tendon thickness in later remodelling stages of partial rupture tendinopathy.

5.1.4 LLLT IN TENDINOPATHY - ANIMAL MODELS

Human tendinopathies have a multifactorial aetiology, and research in human tendinopathies is complex with ethical limitations on evaluation methods (Cook and Khan, 2007). Animal models can never be fully fledged models for human tendinopathies, but they may be useful in reproducing some aspects of human tendinopathy. Behaviour and nutrition can be controlled more easily in animal studies than in human studies. This gives animal studies an edge in being particularly suitable for single-factor experimental design. Another advantage of animal models is that they allow full tissue evaluation of biomechanics properties, histopathology and morphology; although the latter two were not used in this particular project.

The use of animal models in research on tendinopathies also has its´ limitations. Extrinsic risk factors, like equipment and environmental conditions, are associated with human tendinopathy. These factors are difficult to imitate in animal models and restrain use of animal models as true triggers of tendinopathies in the way they occur

in humans (Lui et al., 2011). There are also obvious anatomical differences between bipeds and quadripeds. This affects the validity of using animal models in tendinopathy research and limits extrapolation of findings in animal studies to human tendinopathies (Warden, 2009; Lui et al., 2011).

One animal model for mimicking tendinopathy has been used by inducing repetitive mechanical stress. This over-use model forces animals to run daily for 1 kilometre in 60 minutes, causing development of changes in cellularity, tendon thickness and some 40% reduction in tensile strength in supraspinatus tendons (Soslowsky et al., 2000). In other studies with this over-use model, findings of inflammation and fibre degeneration have been conflicting. Perry et al. (2005) found significant presence of inflammatory markers, while Archambault et al. (2007) found little inflammatory reactions or degeneration, but a switch in procollagens to type II and III.

The mechanical model where a blunt trauma is inflicted to tendons in a mini-guillotine is fairly new in mimicking tendinopathy. In previous studies, a standardized trauma in the mini-guillotine caused distinct histopathological changes in rat tendons (Fillipin et al., 2005; Salate et al., 2005; Oliveira et al., 2009). But is has been an open question how this may affect biological properties of the tendons. During our pilot testing we achieved differences in UTS between healthy and injured rat Achilles tendons of ~10 N. Nevertheless UTS differences only came to 2-3 N in study III. A new rat study has since our study was performed found that the ability to regain normal UTS following tendon injury decreases with age (1, 3 and 12 months) (Lavagnino et al., 2013). This age-related regeneration ability in rat tendons can possible explains the relative low UTS differences found in our study III as we used younger rats (1-2 months) in study III than in the pilot tests (6-12 months).

5.2 METHODICAL DISCUSSION

In this section the methodological aspects of this project's design, procedures, subjects, instruments, data and statistics will be discussed. Methodological limitations are included in the sub-sections.

5.2.1 DESIGN AND PROCEDURES

The repeated measurements design is a strong design as it is especially sensitive in detecting differences in the same subject between conditions measured. In study I biophysical effects from several doses of LLLT irradiation were compared to a control area in the same body part of the subject, which means that subjects were their own controls. This minimizes the possibility of other factors than LLLT causing the changes in biophysical effects between the two measured areas. In study II the biophysical ability of energy from LLLT irradiation to penetrate through rat skin, was compared at several time-points during exposure.
There is consensus that randomized controlled double blind trial is the best suited experimental design for evaluating effects from an intervention. In study III the effect on edema formation and UTS from active-LLLT versus placebo-LLLT after an acute tendon trauma was investigated. The animals' contralateral healthy tendons served as controls.

Controlled manipulation of a variable enables us to infer something about the causality of the observed changes under study, although the strength of the conclusions depends on the level of control in the studies. The researcher can achieve control by uniformly implementing the independent variable, eliminating extraneous variables from the setting and relating to subjects, ensuring the reliability of measurements, and limiting information provided to themselves and subjects (Domholdt, 2005). All three studies in this project were provided with detailed protocols and standardized procedures. The implementation of the independent variable, irradiation doses (in study I and III) and exposure time (in study II), was specified in advance. Extraneous excitation was reduced by use of a highly controlled environment, subjects as their own controls, and by measuring outcome data at one occasion. Information to subjects and researchers

was limited where possible: Subjects in study I were blinded to the irradiation sequence i.e. active doses and placebo. Researchers in study III were blinded to placebo/active LLLT intervention.

LIMITATIONS TO STUDY I

One limitation to study I was that the irradiation doses were delivered in a fixed sequence. Applying the selected doses in a randomized manner would have revealed if this possible confounder was at play. The skin-contact laser irradiation application technique and the thermographic measurement seemed to be fairly insensitive to operator and observer variability.

Pilot testing of intraobserver and interobserver reliability could have been included. This would have made the study more robust by quantifying the variability and possibly eliminating this potential source of bias. All testing measures of outcome for each subject were done on a single occasion. We could have repeated the test sequences on another day to ensure that an acceptable intrasubject test-retest reliability level was achieved.

LIMITATIONS TO STUDY II

Considering that LLLT is an intervention used in clinical practice on humans, one limitation to study II was irradiation performed in rat skin with an *in vivo* procedure. On the other hand, for clinical problems (*in situ*) an *in vivo* experiment is often considered suitable as a first stage for investigating if a specific mechanism may or may not be at play.

LIMITATIONS TO STUDY III

The intervention of LLLT irradiation in study III was set to half an hour after trauma. In hindsight, it is easy to see that more groups with administration of LLLT at other time-points after an acute trauma could have shed light on the optimal timing of LLLT treatment after a trauma. But these results were unexpected and difficult to foresee. Future experiments with this model should include different time-points for irradiation and different irradiation doses.

Another amendment to the design of study III would be to broaden it with groups treated with the 810 nm, 200 mW MOP laser. By doing so parallels to differences in laser effects found in study I and study II could have been drawn.

The design of study III would have been more robust if we had provided pre-trauma test measurements on RTUS tendon thickness. This would have allowed comparison of pre- and post-trauma measurements on the same tendon, which is a variable of less variability than using our comparison to the contralateral control. Additionally, pathohistological outcome measurements subsequent to the double mini-guillotine trauma would have brought supplementary information about the tissue pathology. But our choice of UTS as outcome measure precluded the possibility for making a histological analysis of the actual tendons.

5.2.2 SUBJECTS

The research question for the Thesis was to look into biophysical and biological effects from LLLT lasers in tissue. The target tissue was the skin in study I and study II, and tendons in study III. The subjects under study act as a proxy for the target population of interest. Researchers use information from a sample of subjects to make some inference about the wider population of interest (McAuley, 2003).

The sampling method, probabilistic sample, involves randomization and is preferable when the researcher hopes to generalize findings from a sample. Nonprobability samples have absence of randomization and tend to have more sampling error than probability samples (Domholdt, 2005). The healthy humans in study I were recruited by nonprobability sampling, as a stratified convenience sample. This recruitment method can limit the generalization of the findings, but when subjects are compared to themselves the results will still yield clinical relevance. Power analysis was not done as any similar studies on thermal effects from LLLT lasers on human skin were not found in the literature, and the expected variation is a crucial factor in power analyses. The limited sample size of forty subjects leads to small numbers in some of the subgroups. This may have caused type II statistical errors in comparisons of subgroups. A type II error typically occurs when the statistical analysis results in no

differences between groups when in reality there is a small but true difference. A larger sample could have shed light on whether the observed trends in age subgroups were occurring at random or not.

In study I we chose the dorsal side of the proximal phalanx of the right index finger for laser irradiation. A consideration regarding the anatomical location used for laser irradiation in study I includes the variety of actual sites for LLLT treatment in clinical practice. Therapeutic lasers are commonly used for treatment of tendons and joint disorders. Several tendons and joints in the hand have been subjected to LLLT in clinical studies (Sharma et al., 2002; Ozkan et al., 2004; Ye et al., 2011). Additionally, skin exposed to sunlight develops adaptation and enhances photo-protection to a higher degree than skin usually covered from sunlight (Alaluf et al., 2002). In this perspective laser irradiation in the skin of the hand can be expected to be absorbed more and penetrate less than in other anatomical locations.

There was a high withdrawal rate (62%) of participants in the dark skin group during irradiation with the 810 nm, 200 mW MOP laser. This weakens the precision of the exact mean temperature increase in dark skin caused by the lasers. However, it can be argued that this value is of purely academic interest and beyond the scope of this project. The important clinical finding is that painful temperature increases occur during LLLT irradiation with a 200 mW, 810 nm laser in dark skin. This also implies that the great absorption of laser energy in dark skin probably makes the remaining penetrating energy insufficient to induce photobiological reactions in deeper tissues. As a curiosity, one participant with dark skin and a research background wanted for his own interest to measure how high a painful increase in skin temperature he could tolerate (relative to the control area). The skin temperature increased by 11.2°C during a dose of 2 J, and 22.3°C during a dose of 6 J (irradiation was interrupted by the investigators and he was not irradiated with doses of 9 J and 12 J).

Animals included in study II and study III are inbred for more than 20 generations, and of inbreeding coefficient >98%. This increases homogeneity of the animals, and reduces intrasubject variability in tissues such as skin and Achilles tendons. Animals were given 10 days of acclimatization in the laboratory before the experiments were started.

In study II *in vivo* rat skin overlaying the gastrocnemius muscles from 34 animals was harvested. Six of these skin flaps were discarded because of human errors in the dissection procedure. As an extension of the results in study I, and with the knowledge we have after completing study II, it would have been of great value for clinical practice to investigate infrared class 3B lasers ability to penetrate human skin in different colours and age groups.
In study III sixteen animals were randomized to two groups of 8 animals. During the experiment there were no waivers. The target tissue in study III was *in situ* and *in vivo* rat Achilles tendons.

5.2.3 INSTRUMENTS

In this project electrophysical devices were used for both intervention and outcome measurements.
Both intervention devices, i.e. the 810 nm, 200 mW MOP laser and the 904 nm, 60 mW MOP laser, had stable MOP during exposure time as measured in study II (Paper II, Table 1). The active LLLT probe in study III was measured for control subsequently to study III with the same MOP stability as the probe used in study I and study II.

For the measurements of biophysical and biological effects from LLLT in tissue we used other electrophysical devices. The biophysical effects in skin were measured as heat by the Flir system, ThermaCAM S65HS, and as the amount of penetrated energy by the Optical Power Meter system. The biological effect in tendons were measured as tendon thickness (including the peritendon) by a RTUS device manufactured by General Electric, named Logiq e, fitted with a 12 MHz linear probe. The biomechanical testing of tendon UTS was performed with a pulling devise called MTS-820.

THERMOGRAPHIC IMAGING

Thermographic imaging has been used in medicine since the sixties. Technological improvements during the eighties and nineties have made thermographic cameras more accurate and made this type of imaging less time consuming. Today's

thermographic cameras have a small error rate of only ± 2% and typical precision with deviation of less than 0.1°C when measuring skin temperatures (Villaseñor-Mora et al., 2009; Jiang et al., 2005). One limitation for thermography used as an outcome measurement in medical science has been the adaption of biological systems and the method's inherent sensitivity to environmental temperatures. Consequently, thermographic imaging should be performed in a temperature controlled environment where ambient temperature during measuring sessions does not vary more than 1°C (Jiang et al., 2005). In study I the laboratory temperature varied less than 0.5°C during the period of data collection.

Materials have different abilities to emit thermal radiation, expressed as a material's emissivity with range from 0 (not emitting) to 1 (complete emitting). Thermographic cameras visualize energy emitted from materials, and they are most sensitive to temperature changes in materials with high emissivity. Human skin on the back of the hand has an emissivity of 0.98, and skin pigmentation does not affect this value (Ring, 2006; Villaseñor-Mora et al., 2009). The Flir ThermaCAM S65HS used in this project is calibrated once a year against a blackbody, emissivity ε=0.99, distance=1 meter. Calibration is based on the International Temperature Scale (ITS-90) and performed at the Swedish National Testing and Research Institute.

There are no studies found in the literature on reliability tests of the ThermaCAM S65HS.

Optical Power Meter

The Optical Power Meter in study II was a ThorLabs model PM100 fitted with a S121B optical sensor. The wavelength-specific measurement mode was operating at five specific wavelengths; 635, 670, 780, 810 and 904 nm. For our experiment, the two wavelengths (810 nm and 904 nm) that corresponded to the LLLT lasers were used. Laser power meters are known for having high stability and low measurement errors, but earlier equipment has been flawed by variations in the absolute values obtained during laser radiant power measurements (Nussbaum et al., 1999). For the purpose of study II, we were less interested in the absolute radiant power of the lasers, but more in the stability of the laser MOP and their relative loss of energy over time when passing through the skin barrier.

REAL TIME ULTRASONOGRAPHY

In a previous study from our research group, we found that intrasubject side differences in tendon thickness on RTUS were more reliable than intersubject variations in tendon thickness, as measures of tendon disorders (Bjordal et al., 2003a). The RTUS device used in this project, a GE Logiq e, with a 12 MHz linear probe, has present high interrater reliability and intrarater reliability in Master-thesis on human shoulder tendons thickness (Ingebrigtsen, 2012; Naterstad, 2012) and on median nerve cross-sectional area in human carpal tunnel (Hummelsund, 2008).

ULTIMATE TENSILE STRENGTH

Biomechanical testing of UTS is an evaluation method which has been commonly used in experimental tenotomy studies (Forslund and Aspenberg, 2001; Forslund et al., 2003; Dimmen et al., 2009). Nevertheless UTS testing after tendon trauma induced by a mini-guillotine has, to our knowledge, not previously been reported in the literature. A major technical challenge in UTS testing of healthy rat Achilles tendons has been to obtain a reliable, non-sliding grip of the proximal end of the tendon. We have only found two papers investigating UTS of normal tendons, possibly because investigators have experienced grip slippage and consequently test failure before the tendon ruptured.

During UTS testing in study III most tendons ruptured a few mm from the distal grip zone with forces up to 75.2 N. In this model even healthy rat Achilles tendon UTS can be measured. One aspect of the results in study III is the relatively wide standard deviation (SD) we found for UTS in rat Achilles tendons. Whether this SD reflects the natural variation of rat Achilles UTS or is a consequence of the procedures used for UTS testing is as yet unclear.

5.2.4 DATA

The photo-protection ability of the skin barrier is of relevance when exploring effects from LLLT. The energy from LLLT has to pass the skin barrier to influence physiological processes in underlying tissues. Melanin is a chromophore in human skin which provides photo-protection from sunlight by absorbing photons (Giacomoni, 1995; Taylor, 2002), and with a thermal relaxation time of ~1 μsec (Stratigos and Dover, 2000). The protective role of melanin against UV radiation is well known (Kaidbey et al., 1979; Costin and Hearing, 2007; Brenner and Hearing, 2008), but the specific characteristics by which the skin filters infrared irradiation are poorly described. The photon protective processes in skin include absorption and conversion of irradiated energy to heat energy.

The biophysical parameters as heat and skin penetration time-profile are of scientific value as no studies so far have dealt with these effects from infrared class 3B lasers. Even though heat induction in skin is an indirect effect of LLLT irradiation, this parameter still provides information about differences related to skin colour, age and gender (study I). The amount of energy penetrating skin during exposure is an essential figure in customizing doses for treating tissue beneath the skin (study II).
The biological parameter edema provides a rough estimate of the ongoing processes after an acute tendon trauma. When trauma is followed by treatment (placebo-/active LLLT) the difference in edema between the two groups will reflect the active ingredient of LLLT. The biomechanical parameter UTS is of interests as tensile strength reflects tendons' main *in situ* properties (study III).
It is interesting to note that increased tendon thickness is exhibited irrespective of the state of the tendinopathy being in an acute or chronic phase. In the acute stage of tendinopathy, edema may be present in the paratenon, tenosynovia or intratendinous with loosening cross-links but without defects in the longitudinal tendon structure (Couppe et al., 2009). Increased tendon thickness may also be related to the formation of intratendinous oedema after overload or trauma in a partly degenerated tendon (Kongsgaard et al., 2005).

5.2.5 STATISTICS

The selection of Microsoft Excel 2007 for statistical analysis in study I and study II was based on this software's advantage in graphic and table construction compared to other statistical packages. Excel is claimed to be untrustworthy as a statistical tool. A verification of statistical analysis in SPSS ver.19 confirms that p-values in Students t-tests became slightly more significant from SPSS in both study I and study II. All other SPSS analyses on data in study I-III gave identical results as in Excel.

Generalized Estimating Equation (GEE) used in study II is a strong statistical analysis to estimate the parameters of a generalized linear model with an unknown correlation between outcomes. Since our novel study II on LLLT laser's skin penetration time-profile outcomes was unknown, this statistical method of analysis was suitable.

5.3 FURTHER RESEARCH

Although some aspects of the research question were answered, several new questions have been raised:

- To what extent does the irradiation mode affect the skin penetration profile for infrared class 3B lasers?
- Which laser parameter(s) cause photobleaching effects in skin?
  (The phenomenon observed from the superpulsed 904 nm laser, in study II)
- When is optimal timing for initiation of LLLT after a trauma?
- Do tendons in young and old subjects' respond differently to LLLT?
- How is the infrared-class-3B laser's ability to penetrate human skin?
  (Human skin in different groups of age and skin colour).

Other questions which arose were:

- How are the histopathological findings in young compared to old rat tendons after a double trauma in the mini-guillotine?

5.3.1 FOLLOW-UP STUDIES

As an extension of study II the time-profile for skin penetration for the irradiation mode chopped-pulsed (150 Hz) from the 810 nm, 200 mW MOP laser was investigated. The protocol from study II was used, and irradiation was done with the probe in skin contact (n=25) and not in skin contact (n=19).

- The skin penetration time-profile for chopped-pulsed irradiation was similar to the one for continuous irradiation: 22% (SD ±3.6) of MOP penetrated, and the penetration rate was stable over time.

Another follow-up study was done on timing the initiation of LLLT in acute Achilles trauma. The same dose as in study III was irradiated two hours after trauma. The study also included groups with a dose of 0.5 J half an hour after trauma and 0.5 J two hours after trauma. The same procedures as in study III was applied and tendon thickness on RTUS was the outcome measure. Groups consisted of 8 animals. The results were as follow:

- The LLLT dose of 3 J was irradiated 2 hours after each trauma.
  On day three the tendon thickness was (incl. peritendon) 0.72 mm (SD ±0.11).
- The LLLT dose of 0.5 J was irradiated half an hour after each trauma.
  On day three the tendon thickness was (incl. peritendon) 0.84 mm (SD ±0.09).
- The LLLT dose of 0.5 J was irradiated 2 hours after each trauma.
  On day three the tendon thickness was (incl. peritendon) 0.70 mm (SD ±0.07).

These results indicate that timing the initiation of LLLT after acute tendon trauma is an issue to consider in clinical practice, as edema formation is more prominent from irradiation half an hour after trauma than from irradiation two hours after trauma.

The irradiation dose also had effect on edema formation in rat Achilles tendons, where an irradiation dose of 3 J led to more edema formation than the 0.5 J dose. This indicates that 3 J might be too high a dose in acute trauma on small rodents.

5.4 CONCLUSIONS

The endeavour of the three studies in this Thesis was to investigate some biophysical and biological effects from infrared class 3B lasers.

The null hypothesis in study I on thermal effects in human skin from infrared class 3B lasers when irradiated with high doses was accepted, and the alternative hypothesis of no thermal effects was rejected.
The thermal effects in skin from typical WALT recommended LLLT doses for tendinopathies are negligible (<1.5°C) in light and medium coloured skin. However, higher LLLT doses delivered with a strong class 3B infrared laser (200 mW), are capable of inducing photothermal effects exceeding the thermal pain threshold for humans with dark skin. We found no significant differences in skin temperature between genders or between age groups during the laser irradiation sequence.

The null hypothesis in study II on a fraction of optical energy from infrared class 3B lasers penetrating rat skin was accepted, and the alternative hypothesis of no optical energy penetrate was rejected.
LLLT penetration through rat skin has been shown to provide sufficient optical energy at the sub-dermal level to influence pathological processes and tissue repair beneath the skin. The finding that energy from the superpulsed 904 nm laser penetrate 2-3 times better through rat skin than energy from the continuous 810 nm laser, corresponds well with results of LLLT dose analyses in systematic reviews of LLLT in musculoskeletal disorders. This may explain why the differentiation between these two laser types was needed in the clinical dosage recommendations of WALT.

The null hypothesis in study III on effect from a single LLLT dose in acute tendon trauma was accepted, and the alternative hypothesis of no effects from a single LLLT dose was rejected.
Irradiation with a dose of 3 J within half an hour after a tendon trauma embodies active integrant from LLLT in the ongoing processes in tissue beneath the skin. Optimal timing of LLLT administration after a trauma has not been debated in the literature. Our results show increased edema following LLLT irradiation administrated at half an

hour after trauma. This possibly indicate that half an hour after trauma is too early to initiate LLLT, or that recently LLLT-treated injured tendons may be more vulnerable than untreated tendons to a repeated injury. Another possibility is that the dose simply was too high. Future studies are needed to determine optimal timing of LLLT irradiation and optimal doses in acute tendon traumas.

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Paper I

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Original Research

The Thermal Effects of Therapeutic Lasers with 810 and 904 nm Wavelengths on Human Skin

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Abstract

Objective: To investigate the effect of therapeutic infrared class 3B laser irradiation on skin temperature in healthy participants of differing skin color, age, and gender. ***Background:*** Little is known about the potential thermal effects of Low Level Laser Therapy (LLLT) irradiation on human skin. ***Methods:*** Skin temperature was measured in 40 healthy volunteers with a thermographic camera at laser irradiated and control (non-irradiated) areas on the skin. Six irradiation doses (2–12 J) were delivered from a 200 mW, 810 nm laser and a 60 mW, 904 nm laser, respectively. ***Results:*** Thermal effects of therapeutic LLLT using doses recommended in the World Association for Laser Therapy (WALT) guidelines were insignificant; below 1.5°C in light, medium, and dark skin. When higher irradiation doses were used, the 60 mW, 904 nm laser produced significantly ($p < 0.01$) higher temperatures in dark skin (5.7, SD ± 1.8°C at 12 J) than in light skin, although no participants requested termination of LLLT. However, irradiation with a 200 mW, 810 nm laser induced three to six times more heat in dark skin than in the other skin color groups. Eight of 13 participants with dark skin asked for LLLT to be stopped because of uncomfortable heating. The maximal increase in skin temperature was 22.3°C. ***Conclusions:*** The thermal effects of LLLT at doses recommended by WALT-guidelines for musculoskeletal and inflammatory conditions are negligible (<1.5°C) in light, medium, and dark skin. However, higher LLLT doses delivered with a strong 3B laser (200 mW) are capable of increasing skin temperature significantly and these photothermal effects may exceed the thermal pain threshold for humans with dark skin color.

Introduction

During laser irradiation, photons are transferred from a laser source to the human body. Photons are elementary particles with electromagnetic energy measured as optical output power.[1] Lasers with wavelengths in the near-infrared and red spectrum of light are used in Low Level Laser Therapy (LLLT). Class 3B lasers used in LLLT have a mean output power (MOP) ranging from 5 to 500 mW.

Early reports regarding LLLT speculated that clinical effects were induced by increased temperature, although some authors claimed that the effects of LLLT radiation were induced by athermic physiological processes.[2–3] More recently, research suggests that LLLT effects are produced by photochemical and photobiological processes.[4–6] However, in order to induce these therapeutic processes, light energy must not be completely absorbed by the skin and needs to reach subcutaneous tissue. Energy is transformed and stored as heat during the process of absorption and the amount of heating is inversely related to the penetration ability of the light.

Interestingly, there is a lack of research into the thermal effects from commonly used LLLT devices. A search with the terms "thermal effect", "human skin," and "laser" in PubMed, Embase, Cinahl, and ScienceDirect (October 2009) yielded no references for class 3B lasers.

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Skin color may affect absorption and skin temperature during laser irradiation. Nussbaum[7] found a tendency for infrared LEDs (Light-Emitting Diodes) to produce higher temperatures in human skin folds of participants with dark skin than in those of participants with light skin. Whether skin color influences skin temperature from therapeutic infrared class 3B lasers has not been previously investigated.

The structure, appearance, and biophysical properties of human skin differ between individuals according to their age, sex, and race. The ageing process leads to loss of collagen and elastin fibers, and reductions in the size of epidermal, dermal, and subcutaneous layers contribute to fragility, loss of laxity, and a dry, fine, wrinkled appearance of the skin.[8–11] Genetic and hormonal influences affect the sun-sensitivity of skin. Females have greater sun-sensitivity than males.[12–13] After menopause, female skin thickness decreases due to lower levels of estrogen.[14–15]

Skin color evolved in relation to the amount of sun irradiation in different parts of the world, with populations living close to the equator having darker skin than populations living close to the poles. Human skin color is determined by type and amount of melanin in the skin[16–19] which has a central role in skin photoprotection.[17,20] Thus, differences in skin color and properties are factors that may influence absorption and production of heat in human skin during LLLT irradiation.

Human skin temperature can be precisely and reliably measured with thermographic cameras.[21–25] The advantages of thermographic cameras are that they are non-invasive and can be used without being in contact with the skin. The aim of this placebo-controlled study was to investigate the effect on skin temperature in healthy volunteers of differing skin color, age, and gender during irradiation with doses recommended in the World Association for Laser Therapy (WALT) guidelines from two commercially available therapeutic infrared class 3B lasers. A second purpose was to investigate whether irradiation from therapeutic infrared class 3B lasers can produce thermal effects in human skin.

Ethical approval

The study was given ethical approval by REK Vest in Norway (Ref.no: 091.08). Informed consent was obtained from all participants.

Methods

Subjects

A sample of 40 healthy adult volunteers was recruited and divided by gender, age, and skin color. Participants were stratified according to skin color using Von Luschan's chromatic scale,[26] which ranks color from 1 (= lightest skin) to 36 (= darkest skin). Three arbitrary categories were used: 1 to 15 = "light" skin, 16 to 28 = "medium" skin, and 29 to 36 = "dark" skin. Participants were also stratified according to age as under 40 years of age, between 40 and 60 years of age, and over 60 years of age on the basis of changes in skin optical properties.[11, 27] Individuals with a history of skin disease were excluded.

Instruments

Skin temperature was measured by a thermographic camera (Flir System, ThermaCAM S65HS, Boston, MA) and ancillary software (ThermaCAM Researcher Pro 2.8 SR-1). This software includes tools to quantify the recorded temperature. The camera measures temperatures with a precision of 50 mK at 30°C, and has an accuracy of ± 2% (Prod. information). The Flir ThermaCAM S65HS used in this study is calibrated once a year against a blackbody, emissivity $\varepsilon = 0.99$, distance = 1 meter. Calibration is based on the International Temperature Scale (ITS-90)[28] and performed at the Swedish National Testing and Research Institute. Two commercially available therapeutic lasers were used for irradiation as follows: (a) 810 nm wavelength laser (Thor-DD, London, UK), operated in continuous mode with a mean output power (MOP) of 200 mW, spot size of 0.0314 cm^2 and power density of 6.37 W/cm^2, (b) 904 nm wavelength laser (Irradia, Stockholm, Sweden) operated in pulsed mode with a peak power of 20 W, pulse width of 200 ns (10^{-9} s), and frequency of 700 Hz; and with an MOP of 60 mW, spot size of 0.0364 cm^2, and power density of 1.67 W/cm^2.

Experimental procedure

In order to acclimatize skin temperature, participants remained in the laboratory for 15 min before the experiment started. Participants were instructed that during irradiation they should report to the investigator (a) if they felt any heat sensation in the irradiated area, and (b) if the heat sensation became so uncomfortable that they wanted laser irradiation to be ceased. During the experiment participants sat with their hands on a towel placed on a table (Fig. 1).

Recommended irradiation doses according to WALT guidelines are 2 J for 904 nm lasers and up to 6 J for 810 nm lasers.[29]

Laser irradiation was performed on the proximal phalanx of the index finger. A neighboring area on the proximal phalanx of the same index finger was used as the control area (Fig. 2).

FIG. 1. Laboratory setup. Thermographic camera placed approximately 25 cm over the subject's hand, with the participant sitting at a table with both hands on a towel.

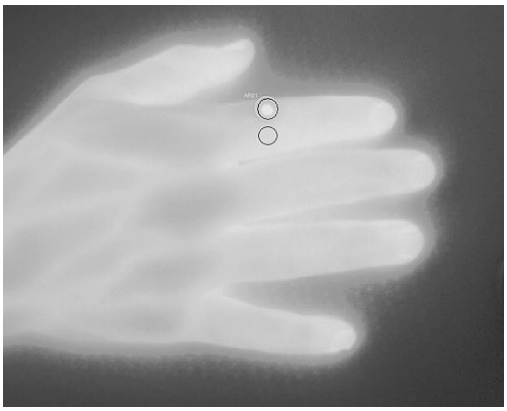

FIG. 2. Thermographic picture (original picture is in color) of the hand after laser irradiation with a dose of 12 joules. Increasing whiteness corresponds to increasing temperature.

One investigator (JHD) operated the thermographic camera, and another investigator (JJ) administered laser irradiation. Each experiment lasted 55 min, with a total of 13 measurements of skin temperature taken from each participant. The first measurement was taken before any irradiation (i.e., baseline). This was followed by six measurements during treatments with different doses of irradiation from the 904 nm laser followed by six measurements during treatments with different doses of irradiation from the 810 nm laser. The sequence of the doses was the same for both lasers, as follows: (a) 2 Joule (J) = 33 s duration from a 60 mW, 904 nm laser and 10 s duration from a 200 mW, 810 nm laser; (b) placebo = 33 s duration from an inactivated 60 mW, 904 nm laser and 10 s duration from an inactivated 200 mW, 810 nm laser (i.e., same duration as 2 J); (c) 6 J = 100 s duration from a 60 mW, 904 nm laser and 30 s duration from a 200 mW, 810 nm laser; (d) 9 J = 150 s duration from a 60 mW, 904 nm laser and 45 s duration from a 200 mW, 810 nm laser; (e) 12 J = 200 s duration from a 60 mW, 904 nm laser and 60 s duration from a 200 mW, 810 nm laser; (f) placebo = 200 s duration from an inactivated 60 mW, 904 nm laser and 60 s duration from an inactivated 200 mW, 810 nm laser (i.e., same duration as 12 J). Placebo irradiation was delivered using the same laser device as the active interventions but the laser was not switched on. Participants were not aware of this fact. During irradiation, the laser probe was held in a position approximately 10 degrees from vertical, and it was stationary in contact with one spot of skin overlying the dorsal side of the proximal phalange of the index finger. Between each laser irradiation there was a 3 min pause. Participants and the operator of the thermographic camera were blinded to active or placebo treatment condition.

Thermography was recorded during the final 5 s of each irradiation dose and continued for 1 min after the end of irradiation (i.e., a total of 1 min and 5 s). The maximum temperatures from the irradiated area and a control area ulnar to the irradiated area on the same index finger were simultaneously registered by ThermaCAM (Fig. 2).

Main outcome measure

The difference in skin temperature between the irradiation surface area and the control area was calculated for each irradiation dose and each laser.

Statistical analysis

Microsoft Excel (Microsoft Office Excel 2007) was used for statistical analysis and graphics. Student's pairwise T-tests and ANOVA were used for comparison of statistical differences with a significance level set at $p < 0.05$. Pearson's correlation coefficients were calculated to determine relationships between irradiation doses and skin temperature.

Results

Forty volunteers expressed interest in taking part in the study, provided written consent, and were included (Table 1).

The baseline skin temperature after acclimatization was 30.7°C (SD ± 2.8) in the proximal part of the index finger.

Placebo irradiation with a treatment time corresponding to the 2 J conditions changed mean skin temperature in the irradiated area by − 0.9°C (SD ± 0.7) for the 60 mW, 904 nm laser, and − 0.2°C (SD ± 1.2) for the 200 mW, 810 nm laser. Placebo irradiation corresponding to the 12 J treatment conditions changed mean skin temperature by 0.4°C (SD ± 1.2) for the 60 mW, 904 nm laser, and 0°C (SD ± 1.1) for the 200 mW, 810 nm laser. There were no significant temperature differences between the irradiated and control areas on the index finger (no p value less than $p < 0.05$).

The tip of the laser probe was in skin contact during laser irradiation, and the probe tip temperature was also measured immediately after irradiation. The probe tip temperature ranged from 31.8°C (SD ± 1.8) to 32.6°C (SD ± 2.0) for the 200 mW, 810 nm laser and from 30.1°C (SD ± 2.2) to 33.7°C (SD ± 3.4) for the 60 mW, 904 nm laser for the irradiation doses from 2 to 12 J (Table 2).

All participants completed the six irradiation doses with the 60 mW, 904 nm laser. Participants with light and medium skin completed six irradiation treatments with the 200 mW, 810 nm laser. However, 8 of 13 participants with dark skin withdrew consent to continue the experiment because they felt uncomfortable heating in the irradiated area with the 200 mW, 810 nm laser. Two of these participants withdrew consent during a 6 J irradiation dose (as recommended by WALT), four withdrew consent during a 9 J irradiation dose, and two withdrew during a 12 J irradiation dose.

Skin temperature at the time of withdrawal showed a large variation between participants. The participant with the highest measured increase in skin temperature (relative

Table 1. Number of Participants Grouped by Age and Skin Color

	Skin color			
	Light	*Medium*	*Dark*	*Total*
Under 40 years	5	5	7	17
From 40 to 60 years	4	7	4	15
Over 60 years	4	2	2	8
Total	13	14	13	40

Table 2. Low Level Laser Therapy Probe Tip Temperature Immediately After Irradiation Doses

	60 mW, 904 nm		*200 mW, 810 nm*	
	Mean	*SD*	*Mean*	*SD*
Placebo	30.2	±2.7	32.0	±1.7
2J	30.1	±2.2	31.8	±1.8
6J	31.2	±1.4	31.9	±2.2
9J	32.8	±2.9	31.8	±2.9
12J	33.7	±3.4	32.6	±2.0

to the control area) during irradiation with the 200 mW, 810 nm laser was a male with dark skin aged 45 years. The skin temperature increased by 11.2°C during a dose of 2 J, and 22.3°C during a dose of 6 J (irradiation was interrupted by the investigators and he was not irradiated with doses of 9 and 12 J). The subject and experimental data related to withdrawals are summarized in Table 3.

There was a positive correlation between increasing irradiation doses and increased skin temperature for both lasers and all skin color groups (Pearson's $r = 0.98 - 0.99$, withdrawals omitted from analysis).

For light and medium skin, no significant thermal effects ($p \geq 0.16$ for any comparisons) were observed when irradiating at doses recommended in the WALT guidelines[29] (i.e., 2 J for 904 nm, and 6 J for 810 nm), with temperatures increasing less than 1.5°C regardless of laser type. However, highly significant increases ($p < 0.0001$) in skin temperature were observed when irradiating dark skin: 6.1°C (SD ± 3.2) for the 2 J dose and 9.2°C (SD ± 4.5) for the 6 J dose, using the 200 mW, 810 nm laser. Also, irradiation with a 2 J dose using the 60 mW, 904 nm laser induced a lower but nevertheless significant ($p = 0.043$) increase in skin temperature, 1.9°C (SD ± 1.8), in dark skin (Figs. 3 and 4).

During the four active irradiation doses from LLLT, the temperature change in dark skin was significantly higher than that in light skin for all irradiation doses, regardless of laser type ($p < 0.01$). During these four doses, delivered with the 60 mW, 904 nm laser, the mean temperature difference between the irradiated area and the control area ranged from − 0.1°C (SD ± 1.0) to 4.0°C (SD ± 1.3) in light skin, from 0.7°C (SD ± 0.9) to 5.3°C (SD ± 1.7) in medium skin, and from 1.9°C (SD ± 1.8) to 5.7°C (SD ± 1.8) in dark skin (Fig. 3). During irradiation with the 200 mW, 810 nm laser, thermal effects were small in light skin, ranging from 0.3°C (SD ± 0.8) to 1.5°C (SD ± 1.2), and moderate in medium skin, ranging from 0.7°C (SD ± 1.4) to 3.3°C (SD ± 1.2). In dark skin, thermal effects were substantial, from 6.1°C (SD ± 3.2) to 9.8°C (SD ± 2.3) across doses from 2 to 12 J (Fig. 4).

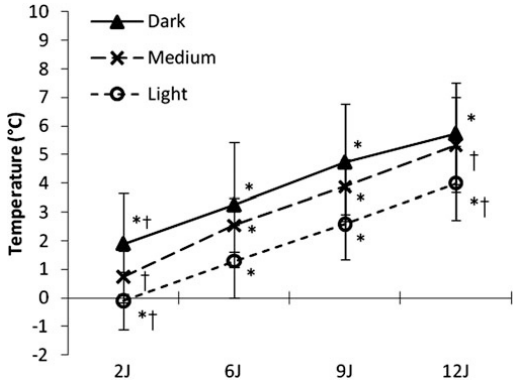

FIG. 3. Doses in joules plotted against temperature change in the three skin color groups during irradiation with the 60 mW, 904 nm laser ($n = 40$). The change in temperature was defined as the difference between temperatures in the irradiated area (laser spot) and a control area 1 cm ulnar on the same index finger (error bars ± SD). *Significant differences between the light-skin group and the dark- and medium-skin groups ($p < 0.01$). †Significant differences between light- and medium-skin groups and the medium- and dark-skin groups ($p < 0.05$).

When equivalent irradiation doses, of 6 J or more, were administered to light and medium skin, there were larger increases in skin temperature for the 60 mW, 904 nm laser when compared with the 200 mW, 810 nm laser ($p < 0.05$) (Table 4).

Due to the high withdrawal rate (62%) of participants in the dark-skin group during irradiation with the 200 mW, 810 nm laser, comparison of skin temperature for the two lasers was problematic in this group.

Table 3. Skin Temperature Thresholds at Withdrawal

Laser data		*Biographical data*			*Temperature data*		
nm	*Dose*	*Skin*	*Sex*	*Age*	*Irradiated*	*Control*	*Difference*
810	6 J	Dark	F	43	35.1	33.2	1.9
810	6 J[a]	Dark	M	45	53.4	31.1	22.3
810	9 J	Dark	F	39	40.3	32.6	7.7
810	9 J	Dark	M	37	41.5	33.1	8.4
810	9 J	Dark	M	41	40.9	33.1	7.8
810	9 J	Dark	M	61	43.1	31.5	11.6
810	12 J	Dark	F	34	40.0	29.2	10.8
810	12 J	Dark	M	42	37.3	32.5	4.8
				Mean	41.5	32.0	9.4

[a]Participant completed irradiation with the 6 J dose (and did not start the 9 J dose).

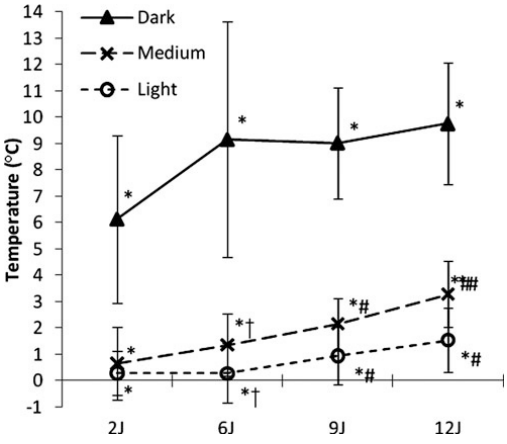

FIG. 4. Doses in joules plotted against temperature change in the three skin color groups during irradiation with the 200 mW, 810 nm laser ($n = 40$). The change in temperature was defined as the difference between temperatures in the irradiated area (laser spot) and a control area 1 cm ulnar on the same index finger (error bars ± SD). * Significant differences between the dark-skin group and the medium-and light-skin groups ($p < 0.01$). † Significant differences between the medium-skin group and the light-skin group ($p < 0.05$). [a)] Six and [b)] eight of thirteen participants with dark skin withdrew because of uncomfortable heating in the LLLT-spot area.

In contrast to the results in light and medium skin, identical doses of laser irradiation resulted in significantly higher skin temperatures in dark skin ($p \leq 0.001$) for the 200 mW, 810 nm laser. Irradiation doses of 2 and 6 J with the 200 mW, 810 nm laser induced a threefold increase in temperature in dark skin when compared with the 60 mW, 904 nm laser (Figs. 3 and 4).

There were no significant differences in skin temperature between males and females during laser irradiation when using doses at or above those recommended by WALT (no p-value lower than $p \geq 0.24$). Adjusting for skin color by removing dark-skin data from the analyses did not affect this result (Figs. 5 and 6).

There was a tendency toward higher skin temperatures in the irradiated area in younger participants (under 40 years of age) for both lasers, although comparisons with older participants just failed to reach statistical significance ($p \geq 0.06$). There was evidence that there were larger differences for higher doses with the 200 mW, 810 nm laser than for doses recommended by WALT, and that the differences persisted when dark-skin data were removed from the analysis (Figs. 7 and 8).

Discussion

In this study, we investigated the effects of irradiation using two commercially available class 3B lasers on human skin temperature for subjects of different skin color, age, and gender. In the early days of LLLT, it was speculated that treatment effects were of thermal origin and similar to heat therapy.[30] However, research in the 1980s supported claims that doses used in clinical practice were too low to produce noticeable thermal and biological reactions in the human body.[31] Subsequent research in the 1990s into the biophysical properties of LLLT irradiation suggested that the therapeutic effects of LLLT irradiation resulted from non-thermal mechanisms.[32] In recent years, more powerful class 3B laser devices have arrived on the market, with laser manufacturers claiming that "more powerful is better".

Thermoregulation of the skin is mainly controlled by the hypothalamic nuclei. Heating of small areas of skin by conduction results in activation of skin thermoreceptors and leads to the release of nitric oxide which acts directly on blood vessels to dilate the vessels and thereby regulate body temperature.[33–36] Central thermoregulation acts to regulate changes in larger areas of the body, such as the entire index finger, and cannot result in local temperature differences like those we found between the two measurement areas on the proximal phalanx of the index finger.

We observed a consistent thermal effect of laser irradiation, which was strictly limited to the area of the irradiation spot. Skin temperature decreased to that of the surrounding areas within 15 s after irradiation had ceased. We interpret this effect as laser light absorption and subsequent heating of the skin, because it was clearly limited to the area of irradiation and did not spread to the control site, which was 1 cm away from the irradiated site on the same index finger. We also observed an increase in skin temperature which started proximally in the hand and then spread to the fingers in several participants following irradiation with 6 J (100 s) or more from the 904 nm laser. Interestingly, the temperature difference noted between the irradiation

Table 4. Temperature Changes in Light and Medium Skin During Comparable Irradiation Doses from Two Different Lasers

	Light skin			*Medium skin*		
Irradiation dose	*200 mW, 810 nm Mean ± SD (°C)*	*60 mW, 904 nm Mean ± SD (°C)*	*t-test (2-tailed) p value*	*200 mW, 810 nm Mean ± SD (°C)*	*60 mW, 904 nm Mean ± SD (°C)*	*t-test (2-tailed) p value*
2 Joules	0.3 ± 0.8	−0.1 ± 1.0	0.31	0.7 ± 1.4	0.7 ± 0.9	0.85
6 Joules	0.3 ± 1.1	1.3 ± 1.3	0.04[a]	1.4 ± 1.2	2.5 ± 0.9	0.01[b]
9 Joules	0.9 ± 1.1	2.6 ± 1.2	0.00[b]	2.2 ± 1.0	3.9 ± 1.0	0.00[b]
12 Joules	1.5 ± 1.2	4.0 ± 1.3	0.00[b]	3.3 ± 1.2	5.3 ± 1.7	0.00[b]

[a]Significant ($p < 0.05$) differences during use of different lasers.
[b]Significant ($p < 0.01$) differences during use of different lasers.

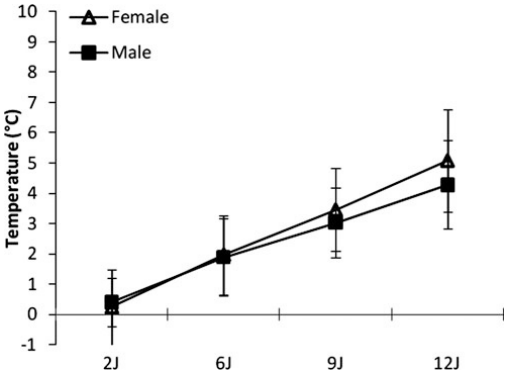

FIG. 5. Doses in joules plotted against temperature change in males and females during irradiation with the 60 mW, 904 nm laser ($n = 27$). The change in temperature was defined as the difference between temperatures between the irradiated area (laser spot) and a control area 1 cm ulnar on the same index finger (error bars ± SD).

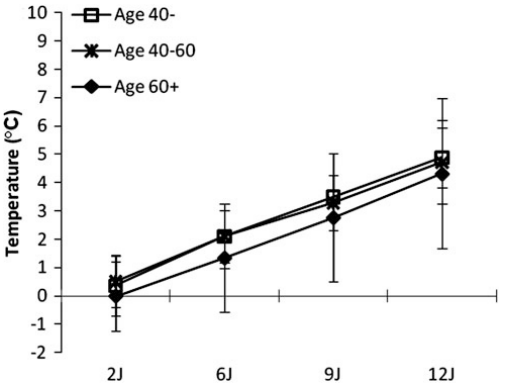

FIG. 7. Doses in joules plotted against temperature change in the three age groups during irradiation with the 60 mW, 904 nm laser ($n = 27$). The change in temperature was defined as the difference between temperatures in the irradiated area (laser spot) and a control area 1 cm ulnar on the same index finger (error bars ± SD).

spot and the control area was statistically significant despite the increase in background temperature across the entire hand. We infer from these observations that laser irradiation initiates central thermoregulatory mechanisms that affect skin circulation in the skin surrounding the area of irradiation. Slow-onset circulatory effects lasting more than 120 s after irradiation have been reported in humans following 2 min of irradiation with 74 mW, 810 nm laser[37] and 20 min of irradiation with a 30 mW, 632 nm laser[38] and in rabbits after 10 min with a 10 mW, 904 nm laser.[39] Evidence also suggests that equal doses below 10 J delivered over a longer period of time stimulates cell activity and modulates inflammation to a greater extent than lasers with higher MOP using shorter irradiation times.[40–41]

Thermal effects in the human skin need to reach a threshold magnitude before they are perceived by individuals. Recent studies have shown that there is spatial summation of warming sensation thresholds in the human hand ranging from 35.4°C (±1.0) when large areas (9 cm^2) are heated to 42.3°C (±3.0) when small areas (2 cm^2) are heated.[42] During irradiation, heat could either stem from the absorption of laser light or transfer of heat energy from the laser probe to the skin by conduction as the laser probe heats during production of the laser beam. Our measurements of

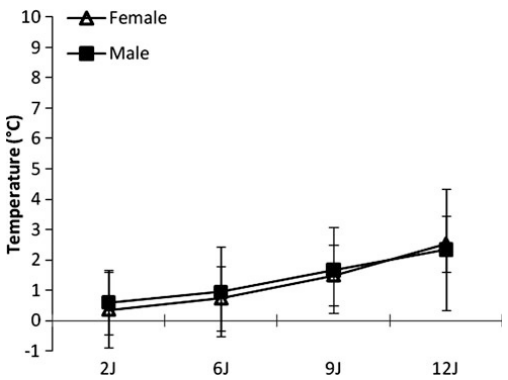

FIG. 6. Doses in joules plotted against temperature change in males and females during irradiation with the 200 mW, 810 nm laser ($n = 27$). The change in temperature was defined as the difference between temperatures in the irradiated area (laser spot) and a control area 1 cm ulnar on the same index finger (error bars ± SD).

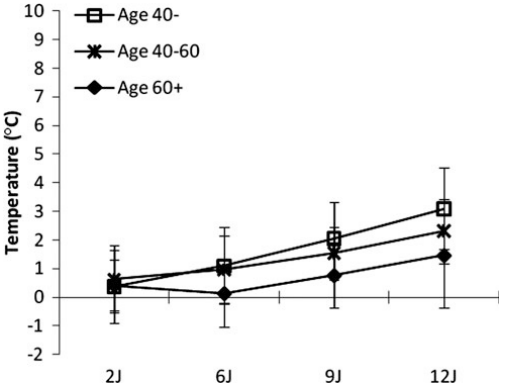

FIG. 8. Doses in joules plotted against temperature change in the three age groups during irradiation with the 200 mW, 810 nm laser ($n = 27$). The change in temperature was defined as the difference between temperatures in the irradiated area (laser spot) and a control area 1 cm ulnar on the same index finger (error bars ± SD).

the temperature of the laser probe tips immediately after irradiation suggest that the probe tip was not responsible for elevations in skin temperature; probe temperatures were below 34°C with doses of 12 J, and below 32°C with doses recommended by WALT.[29] The thermal effects for doses recommended by WALT were below the warmth sensitivity thresholds for human skin for the respective lasers. This is particularly important because the sensation of warmth can compromise blinding of active and placebo laser units in research studies.

Guinot[12] found that female skin is more sun-sensitive than male skin, but we did not find any significant differences in skin temperature between males and females during infrared laser irradiation. Structures and entities of the human skin do change with age and this change could result in modified skin sensitivity to laser irradiation. Even though there was a tendency to more heat production in younger skin (under 40 years of age) than in older skin, we did not find significant differences in skin temperature according to age.

Our results showed that the heat induced in human skin during LLLT irradiation is strongly related to skin color. Skin temperature was higher at all irradiation doses for participants with dark skin than for those in other skin color groups, regardless of laser type. Skin temperature differences for the 60 mW, 904 nm laser were moderate (about 4°C) during irradiation doses from 2 to 12 J, and increased according to dose in a linear manner for all skin color groups. While the dose-temperature profile was linear for light- and medium-skin groups with the 200 mW, 810 nm laser, the induced thermal effects were higher in darker skin. One study using LEDs with an 840 nm infrared wavelength found no significant differences between participants with different skin colors but there were few participants with the darkest skin color in the sample and the power density for the LED source was low and only 1–2% of the power density used in the present study.[7]

The significant heating of dark skin by the 200 mW, 810 nm laser has implications for irradiation safety and clinical practice. There was a three- to sixfold increase in skin temperature in dark skin, reaching 42–43°C, when compared with light and medium skin. This temperature corresponds to the threshold for painful thermal stimuli[43] and was achieved even at small laser irradiation doses.

Both lasers used in this study were classified as class 3B, and their irradiation spot sizes were almost identical, 0.0364 cm^2 (60 mW, 904 nm) and 0.0314 cm^2 (200 mW, 810 nm). The authors see no reason why such a small spot size difference would affect temperature to any appreciable degree. Therefore, it is necessary to consider differences in wavelengths, MOP, and irradiation mode (continuous vs. pulsed) between the lasers as possible causes of the study results. Infrared wavelengths penetrate human skin more readily than red wavelengths.[1, 44] However, there are only minor differences (<20%) in rat skin penetration for wavelengths within the near-infrared spectrum, with 810 nm penetrating slightly more than 904 nm wavelengths.[45] The observed differences in our study seem to exceed what can reasonably be explained by wavelength differences. We expected that the largest increase in skin temperature would occur using the 200 mW, 810 nm laser because its MOP is approximately three times greater than the 60 mW, 904 nm laser, and it has a shorter irradiation time. However, skin temperature increased more in light and medium skin during irradiation with the 904 nm laser. It seems unlikely that a physiological response such as more rapid removal of excess heat should occur during and after irradiation with the stronger 810 nm laser than the 904 nm laser. Future studies are required to investigate in greater detail the influence of MOP and irradiation mode (pulsed vs. continuous) in heat production on human skin during laser irradiation.

There is strong clinical evidence, reflected in the WALT guidelines, that pulsed 904 nm lasers have lower optimal dose ranges in musculoskeletal disorders than continuous 810 nm lasers, possibly because less energy is lost in the skin barrier with the pulsed 904 nm laser.[46] Our results from light and medium skin may be seen as contradictory to this suggestion because equal doses yielded significantly higher skin temperatures with the pulsed 904 nm laser than with the continuous 810 nm laser.

During LLLT in which the thermal effect on the skin is small, the irradiated energy facilitates other photoprocesses in the local tissue. Infrared irradiation stimulates photobiological reactions through photoacceptors that take part in metabolic reactions in the cells.[47–54] As the purpose of irradiation during LLLT is to reach structures beneath the skin layers, the significant thermal response in dark skin raises a question as to whether sufficient energy reaches subdermal target tissue. Our results suggest that the 200 mW, 810 nm laser should probably not be used clinically with doses at or above 6 J in persons with dark skin.

Conclusion

The thermal effects of LLLT at doses recommended in the WALT guidelines for musculoskeletal and inflammatory conditions are negligible (<1.5°C) in light- and medium-colored skin. However, at higher irradiation doses delivered with a strong class 3B laser (200 mW), LLLT is capable of increasing skin temperature significantly, and these photothermal effects may exceed the thermal pain threshold for humans with dark skin. The thermal effects in dark skin were most pronounced during irradiation with the 200 mW MOP, 810 nm laser; 62% of participants withdrew because of uncomfortable heating in the irradiated spot area. There were no significant differences between age groups or between gender groups in skin temperature during laser irradiation.

Author Disclosure Statement

No competing financial interests exist.

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Skin Penetration Time-Profiles for Continuous 810 nm and Superpulsed 904 nm Lasers in a Rat Model

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Abstract

Objective: The purpose of this study was to investigate the rat skin penetration abilities of two commercially available low-level laser therapy (LLLT) devices during 150 sec of irradiation. ***Background data:*** Effective LLLT irradiation typically lasts from 20 sec up to a few minutes, but the LLLT time-profiles for skin penetration of light energy have not yet been investigated. ***Materials and methods:*** Sixty-two skin flaps overlaying rat's gastrocnemius muscles were harvested and immediately irradiated with LLLT devices. Irradiation was performed either with a 810 nm, 200 mW continuous wave laser, or with a 904 nm, 60 mW superpulsed laser, and the amount of penetrating light energy was measured by an optical power meter and registered at seven time points (range, 1–150 sec). ***Results:*** With the continuous wave 810 nm laser probe in skin contact, the amount of penetrating light energy was stable at ~20% (SEM ± 0.6) of the initial optical output during 150 sec irradiation. However, irradiation with the superpulsed 904 nm, 60 mW laser showed a linear increase in penetrating energy from 38% (SEM ± 1.4) to 58% (SEM ± 3.5) during 150 sec of exposure. The skin penetration abilities were significantly different ($p < 0.01$) between the two lasers at all measured time points. ***Conclusions:*** LLLT irradiation through rat skin leaves sufficient subdermal light energy to influence pathological processes and tissue repair. The finding that superpulsed 904 nm LLLT light energy penetrates 2–3 easier through the rat skin barrier than 810 nm continuous wave LLLT, corresponds well with results of LLLT dose analyses in systematic reviews of LLLT in musculoskeletal disorders. This may explain why the differentiation between these laser types has been needed in the clinical dosage recommendations of World Association for Laser Therapy.

Introduction

Lasers are used for a number of different purposes in medicine. Class 4 surgical lasers are used to cut and destroy biological tissue, whereas low-level laser therapy (LLLT) is administered with weaker class 3B lasers and mean output powers (MOP) < 500 mW.[1] The most commonly used lasers in LLLT are gallium-aluminum-arsenide (GaAlAs) with a wavelength of 810 nm (± 50 nm) operating in continuous output mode, and gallium-arsenide (GaAs) with a wavelength of 904 nm superpulsed with high peak power pulses. LLLT emerged as a treatment option in clinical practice approximately three decades ago.[2] After the initial upsurge, LLLT gained a poor reputation because of a string of negative research results being published. This picture has slowly changed again as an increasing amount of evidence is pointing toward the existence of specific therapeutic windows for LLLT. Several studies have found that anticipated optimal doses yield significantly better results in tendinopathies,[3] osteoarthritis,[4] and neck pain.[5]

The skin is the body's external boundary in animals and humans. Among other things, the skin serves as a barrier against physical and chemical intrusions.[6] The skin also represents a barrier to applied physical energy from

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electrophysical agents (EPAs), which represent one of the four treatment pillars of physical therapy.[7]

EPAs are used to treat tissue such as muscles, tendons, ligaments, and joint capsules. This makes the skin barrier of particular interest for research on EPAs. Variation characteristics in the biophysical ability to penetrate skin have direct implications for the dosing of LLLT in clinical settings. EPA therapy dosing has historically been based on clinical experience, rather than solid scientific evidence from basic research.[8]

The skin's photoprotective properties, during epidermal light reflectance and melanocytes light absorption, avoid tissue injury caused by radiation from sunlight.[9,10]. The skin penetration characteristics of ultraviolet and visible light, are fairly well mapped in biophysical and dermatological literature. However, this literature provides only limited data about the penetration ability of the most commonly used infrared and near-infrared LLLT wavelengths in clinical settings.

Biophysically, the ability of lasers to penetrate tissue is dependent upon the laser's wavelength. Light with a wavelength range of 700–1000 nm is infrared and invisible, and penetrates tissue better than light in the red wavelengths (600–700 nm).[11] Clinically, laser irradiation in skin flaps has shown that penetration increases linearly with wavelengths from 450 nm to 1030 nm.[12] This was further supported by results from a study of two different red laser wavelengths in human skin flaps, which showed that a wavelength of 675 nm penetrated better than did a wavelength of 632.8 nm.[13] Other studies have reported similar findings with wavelength-dependent penetration in animal tissue.[14] These results correlate well with another study showing that a greater amount of energy penetrated rabbit skin with a wavelength of 904 nm, than with a red wavelength of 632.8 nm.[15]

In a systematic review of LLLT studies on lateral epicondyle tendinopathies, Bjordal et al.[3] found effective doses from superpulsed 904 nm lasers to be significantly lower than effective doses from 632.8 nm lasers. Guidelines from World Association for Laser Therapy (WALT) have also differentiated between wavelengths. Typically, WALT recommends doses that are twice as high for lasers within the 780–860 nm wavelength range, than for lasers with a wavelength of 904 nm.[16] WALT guidelines doses are derived from some 160 published randomized controlled LLLT trials. The difference in energy dose can only partly be explained by wavelength, which typically accounts for $<20\%$ of the penetration difference.[12] However, a satisfactory causal understanding of the need for differentiated dosing is still lacking. An aspect other than wavelength dependency is the mode of operating the laser. GaAs lasers with 904 nm wavelength operate with strong, short pulses (peak power 10–100 W) in a superpulsed mode with nanosecond or picosecond pulse durations, whereas 780–860 nm GaAlAs lasers typically operate in a continuous mode or with chopped pulses (peak power <0.5 W). This feature has not been extensively addressed in the LLLT literature, and few studies have compared continuous with pulsed and superpulsed irradiation. In a recent article, Hamblin et al.[17] searched the literature for possible differences in therapeutic effects between those of continuous and pulsed laser irradiation. Their conclusion was that pulsed irradiation mode seems to be superior to continuous mode. However, this result could also be caused by differences in laser parameters other than irradiation modes, as no head-to-head comparisons were performed with equal doses. Moriyama et al.[18] compared superpulsed with continuous irradiation mode from 905 nm lasers in acute knee inflammation in mice induced by Zymosan-A, and found more pronounced upregulation of inducible nitric oxide synthase (iNOS) from superpulsed irradiation mode. They suggested that inflammatory pathway responses were activated by different mechanisms in superpulsed and continuous irradiation modes.

The few existing LLLT studies that deal with penetration issues have focused either on energy loss[19,20] or on penetration depth.[13,15] In LLLT treatment, an irradiation dose is typically administered during periods lasting from 20 to 30 sec up to a few minutes. No studies have yet investigated the time-profiles for skin penetration of energy from LLLT devices. With this perspective, we decided to perform a study with two commonly used lasers in LLLT.

Aim

The aim of this study was to investigate the rat skin penetration abilities of two commercially available LLLT lasers during 150 sec of exposure.

Materials and Methods

Materials

Thirty-four matured male Sprague–Dawley rats weighing 250–300 g were housed with 3–4 animals together in individually ventilated cages. Light cycles were kept at 12+/12− h, with water and food *ad libitum*. The animals were then euthanized and 62 skin flaps ($n=62$) overlaying the gastrocnemius muscles on the hind legs were successfully dissected free and irradiated within 3 min after euthanization. Six skin flaps were discarded because of human errors in the dissection procedure.

Instruments

The laser MOP was measured with an Optical Power Meter System (Thorlabs Instruments, U.K.). The Optical Power Meter System consists of a PM100 Display unit with sample rate of 6 Hz and accuracy of $\pm 1\%$, and a S121B silicon sensor. The S121B sensor input had an aperture with diameter of O = 9.5 mm with an optical power range 500 nW–500 mW and an accuracy of $\pm 5\%$ (manufacturer's specification).

Two commercially available therapeutic lasers were used for laser irradiation: (1) 810 nm wavelength laser (Thor-DD, U.K.), operated in a continuous mode with MOP of 200 mW, spot size 0.0314 cm^2, and power density of 6.37 W/cm^2 (manufacturer's specification); and (2) 904 nm wavelength laser (Irradia, Sweden) operated in a superpulsed mode: Peak power 20 W, superpulsed width 100 ns (10^{-9} sec) with a frequency of 6 kHz; and 60 mW MOP, spot size 0.0364 cm^2, and power density 1.67 W/cm^2 (manufacturer's specification).

Experimental procedure, part 1

Step one: energy output measured directly. The two lasers were tested for MOP during 150 sec of exposure with no obstacles between the laser source and the Optical Power Meter.

Step two: energy output measured after penetrating a plastic film. The two lasers were tested for MOP during 150 sec of exposure with a transparent plastic film between the laser source and the Optical Power Meter.

Step three: energy output measured after penetrating freshly harvested rat skin flaps. with the probe in skin contact. The animal was put into gas anesthesia (isoflurane, Isoba), and then given 0.4 mL intraperitoneal anesthesia (Mebumal 50 mg/mL). Hair was shaved from both hind legs.

Experimental procedure, part 2

Animals were then euthanized by cardiac arrest from a 1 mL saturated potassium chloride solution.

The skin overlaying the gastrocnemius muscle was then dissected free: distally from calcaneus, anteriolateral and anteriomedial of the edge of the gastrocnemius muscle, and proximal to the gastrocnemius muscle (Fig. 1).

The Optical Power Meter System was tuned to the same wavelengths as the respective test lasers.

All skin flaps were then irradiated in a sequential manner with increasing exposure times for each laser.

For every skin flap the measurement procedure started with registering the lasers' MOP directly into the Optical Power Meter. A plastic film was then placed over the sensor. The skin flap was then placed on the plastic film and the laser MOP was registered. During the exposure, the laser probe was in full skin contact for 150 sec. The laser probe was held in skin contact with slight pressure necessary to maintain the probe in a fixed position. The therapist sought to apply the same amount of pressure for all skin flaps. Laser penetration through the skin plus plastic film was registered at seven time points; immediately after being activated, after 10 sec, after 30 sec, and then every 30 sec up to 150 sec.

Step four (same procedure as in step three, but with the probe not in skin contact). The laser probe was now kept $\sim$1 mm from the skin during LLLT irradiation.

FIG. 1. A skin flap, consisting of skin overlaying the gastrocnemius muscle.

Main outcome measure

The power was measured in mW without obstacles after penetration through air, plastic film, and rat skin flap, and as a percentage of the measured nominal MOP.

Statistical analysis

Statistical differences were calculated by Statistical Package for the Social Sciences (SPSS, v.19) using repeated measures ANOVA, generalized estimating equations(GEE), for the whole sequence (measurements at 10 sec are omitted in these calculations). Microsoft Excel (Microsoft Office Excel 2007) was used for statistical descriptive analysis, Student's *t*-test *p*-value significant differences, and graphs.

Ethical approval

This study was approved by the local animal laboratory committee at the University of Bergen, Norway (Appl. No. 20102676).

Results

Step one (no obstacle, through air only)

The MOP from both lasers were stable during 150 sec of exposure. The 904 nm laser increased MOP by 0.3 mW (SEM $\pm$ 0.4), whereas the 810 nm laser reduced MOP by 9.8 mW (SEM $\pm$ 1.9) by the end of 150 sec of exposure (Table 1). The statistic GEE estimated a slope of 0.25 mW per 30 sec (95%CI: 0.07 to 0.42, $p < 0.01$) for the 904 nm laser; and a slope of -1.14 mW per 30 sec (95%CI: -1.44 to -0.84, $p < 0.01$) for the 810 nm laser.

Step two (through plastic film)

The plastic film reduced MOP by 3.6%, or 2.3 mW (SEM $\pm$ 1.2), on average from the 904 nm laser; and by 6.6%, or 12.7 mW (SEM $\pm$ 1.0), on average from the 810 nm laser (Table 1). The statistic GEE estimated a slope of 0.16 mW per 30 sec (95%CI: -0.10 to 0.42, $p = 0.23$) for the 904 nm laser; and a slope of -1.00 mW per 30 sec (95%CI: -1.49 to -0.52, $p < 0.01$) for the 810 nm laser.

Step three (through skin, probe in skin contact)

The two lasers had statistically different skin penetration abilities. The percentage of energy penetrating skin from the 810 nm laser was stable at $\sim$ 20% of MOP (ranging from 19.5% [SEM $\pm$ 0.6] to 20.4% [SEM $\pm$ 0.6]) during the exposure period (Table 1). The statistic GEE estimated slope was -0.26% of MOP per 30 sec (95%CI: -0.35 to -0.17, $p < 0.01$), whereas the percentage of energy penetrating skin from the 904 nm laser increased almost linearly during the exposure period, from 38.7% of MOP (SEM $\pm$ 1.4) to 58% of MOP (SEM $\pm$ 3.5) (Table 1). The statistic GEE estimated slope was 2.29% per 30 sec (95%CI: 1.69 to 2.88, $p < 0.01$) for the 904 nm laser.

Comparing the percentage of MOP penetrating skin from the two lasers, there were significant (*t*-test, $p < 0.01$) differences at all measured time points (Table 1 and Fig. 2).

Step four (through skin, probe not in skin contact)

Both lasers showed the same trends of energy penetrating skin as was the case with the laser probes in skin contact. There were significant (*t*-test, $p < 0.01$) differences in percentage of MOP penetrating skin from the two lasers at all

Table 1. Skin Penetration with LLLT Devices. Penetration Time-Profile for Continuous 810 nm and Superpulsed 904 nm Wavelength Lasers in a Rat Model

		Exposure time (in seconds):						
	n	*1*	*10*	*30*	*60*	*90*	*120*	*150*
Direct (through air)								
904 nm (mW)	5	64.7	64.3	64.2	64.4	64.6	64.8	65.0
(SEM±)		0.2	0.2	0.5	0.4	0.4	0.4	0.4
810 nm (mW)	8	198.3	195.5	192.9	191.2	189.7	189.3	189.5
(SEM±)		1.8	2.1	2.0	1.8	1.8	2.2	1.9
Through plastic film								
904 nm (mW)	5	61.6	61.6	62.1	62.8	62.6	62.3	62.6
(SEM±)		1.6	1.4	1.2	1.2	1.0	0.8	0.8
810 nm (mW)	7	185.5	182.8	180.1	178.4	177.4	176.5	175.7
(SEM±)		0.7	0.8	1.0	1.2	1.2	0.9	1.3
Skin penetration (through plastic film and skin)								
Probe in skin contact								
904 nm (% of MOP)	62	38.7	42.0	45.1	47.0	54.7	56.3	58.0
(SEM±)		1.4	1.6	1.8	1.9	3.4	3.5	3.5
810 nm (% of MOP)	62	20.4	20.1	19.9	19.5	19.9	19.8	19.8
(SEM±)		0.6	0.7	0.7	0.6	1.3	1.2	1.3
t-test (*p*)		1.30E-19	1.01E-20	5.58E-21	1.74E-22	3.33E-11	2.70E-11	1.04E-11
Probe not in skin contact								
904 nm (% of MOP)	15	36.8	37.6	38.6	41.2	43.6	43.4	44.4
(SEM±)		2.4	2.8	2.8	3.2	3.3	2.9	3.0
810 nm (% of MOP)	9	25.5	25.5	25.5	24.3	24.4	23.1	23.7
(SEM±)		2.2	2.0	1.8	1.8	1.8	1.7	1.5
t-test (*p*)		3.91E-03	5.92E-03	3.10E-03	1.42E-03	0.46E-03	0.01E-03	0.44E-03

The mean energy output from two commercial LLLT-lasers, during 150 sec of irradiation: direct into an Optical Power Meter, through plastic film, and through plastic film plus skin (with the LLLT-probe in skin contact and not in skin contact).
LLLT, low-level laser therapy; MOP, mean output powers.

measured time points (Table 1), even though the slope of increased penetration during processing time from the 904 nm laser was smaller than with the probe in skin contact. The statistic GEE estimates a slope of 1.42% of MOP per 30 sec (95%CI: 0.82 to 2.03, $p < 0.01$) with the probe not in skin contact. In percentages, the amount of energy penetrating skin increased from 36.8% (SEM ± 2.4) to 44.4% (SEM ± 3.0) during exposure (Table 1 and Fig. 2).

In skin contact versus not in skin contact and probe's shape

For the 810 nm laser, the amount of energy penetrating the skin was slightly higher during irradiation than with the probe in skin contact: The percentage of MOP was ~24% (ranging from 23.1% [SEM ± 1.7] to 25.5% [SEM ± 2.2]) during the exposure period with the probe not in skin contact (Table 1 and Fig. 2).

The statistic GEE estimated slope was −0.42% of MOP per 30 sec (95%CI: −1.27 to 0.43, $p = 0.33$).

Irradiation with the laser probe in skin contact versus not in skin contact resulted in a change in skin penetration for both lasers. This change was most likely not attributed to laser parameters, but to the different physical shape of the two probe tips. The 904 nm laser probe has a protruding convex lens, which squeezes the skin when the probe is in skin contact. This will cause better penetration with full skin

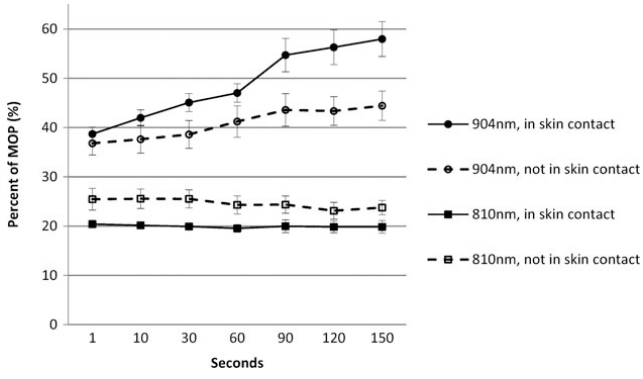

FIG. 2. Percentage of mean output powers (MOP) (±SEM) penetrating skin during 150 sec of irradiation, from the 904 nm (60 mW, superpulsed) laser and the 810 nm (200 mW, continuous) laser. Data are represented with the laser probe in skin contact and not in skin contact.

FIG. 3. Laser probe tip, the 904 nm laser with a protruding lens (*left*), and the 810 nm laser with a folded up lens (*right*).

contact than with no skin contact. On the other hand, the 810 nm laser probe has a recessed flat window. Here, the metallic ring surrounding the lens will push skin underneath the lens, which leads to less penetration with the probe in skin contact than not in skin contact (Fig. 3).

Discussion

The skin barrier has been a major obstacle for EPAs since they were introduced in the treatment of tissue pathology.[21] Questions such as how deep can different energy forms penetrate into the body, and what happens in terms of energy loss in this process has intrigued researchers for decades. LLLT has poor penetration ability when compared with other energy forms such as electromagnetic and ultrasound radiation. Nonetheless, it is important to emphasize that the current study demonstrates that between 20 and 58% of the energy delivered to the skin surface is penetrating the rat skin barrier during LLLT irradiation. The important conclusion is therefore that this residual energy should be more than enough to reach the dose threshold for stimulating physiological and tissue repair processes.

The first two steps of the experimental procedure developed as expected, showing that both lasers delivered stable MOP, and that energy loss in a plastic film was negligible and stable. The time-profiles for skin penetration, during 150 sec of exposure, were, however, distinctly different for the two lasers. For the 810 nm continuous laser, the penetration ability stayed at the same level during 150 sec of exposure, whereas the penetration from the 904 nm superpulsed laser increased in an almost linear manner during 150 sec of exposure. The higher skin penetration ability of the 904 nm superpulsed laser increased from +18% initially to +28% at 150 sec of exposure. This time-profile of increased skin penetration ability during exposure time has, to our knowledge, never been demonstrated before. The pronounced differences cannot be explained by different wavelengths alone. According to studies investigating the ability of different wavelengths in light to penetrate skin, a difference of 10^{22} to 15%,[23] could be expected between the wavelengths 800 nm and 900 nm. This leads us to speculate that the irradiation mode is the most likely source for the observed differences in skin penetration. It has previously been suggested that strong pulses may cause a photobleaching effect in the skin barrier over time.[24]

In the research for identification of possible LLLT dose-response patterns, we suggested already in 2001 that the different laser wavelengths and irradiation modes should be classified in different categories.[25] At that time, the differentiation was governed more by minimizing uncertainty than by solid evidence for these possible differences.

Our current findings contributes to a plausible explanation for different effective doses from 904 nm and 780–860 nm lasers, found in clinical studies and reflected in WALT guidelines.[16] It can be illustrated by an example with two 60 mW MOP lasers, that WALT recommendations of twice as high doses for 810 nm lasers than for 904 nm lasers, as they result in approximately equal amount of energy penetrating the skin. A dose of 4 J from an 810 nm, 60 mW, continuous laser, takes 67 sec to deliver. The skin penetration rate with the probe in skin contact is 20%. The cumulated amount of energy penetrating skin is 0.8 J ($=20\% \times 0.06\,\mathrm{W} \times 67\,\mathrm{sec}$). The delivering of a 2 J dose from a 904 nm, 60 mW, superpulsed laser, takes 33 sec. With the probe in skin contact, skin penetration increase linearly from 38 to 45% during 30 sec of exposure (percentages are in accordance with Table 1). The amount of energy penetrating skin cumulates to 0.82 J

$$\{=[(38\% \times 0.06\,\mathrm{W}) + (\tfrac{1}{2} \times [45 - 38\%] \times 0.06\,\mathrm{W})] \times 33\,\mathrm{sec}\}$$

In line with our currently demonstrated skin penetration profile, Castano et al.[26] found long processing time from superpulsed 810 nm lasers to be more effective than short processing time in inflammatory rat knee arthritis. In Castano's study, all other irradiation parameters were held equal.

In a study with LLLT intervention in induced arthritis in mouse knees, Moriyama et al.[18] found irradiation mode to activate iNOS expression differently. They suggested different mechanisms in activating the inflammatory pathway response as an explanation to the significant differences found between superpulsed and continuous irradiation mode from 905 nm lasers. In perspective of our current findings, the amount of energy penetrating skin from superpulsed lasers is more than double the amount of energy from continuous lasers, during 200 sec of exposure. These circumstances could as well be part of the given reason.

This skin penetration profile, with a linear increase in amount of energy passing skin during an irradiated dose from the superpulsed laser, points out processing time as an interesting parameter in LLLT with superpulsed lasers.

In most clinical studies on animals, LLLT irradiation is initiated hours after an injury or injection. In Moriyama's study with increased inflammatory reactions in mouse knees after LLLT, the treatment from a superpulsed laser was 15 min after induced inflammation. Similarly, we found increased edema in rat Achilles tendon treated with 3 J from a superpulsed laser within 30 min after a blunt trauma.[27] Whether these increased inflammatory reactions are caused

by too early irradiation after induced injury, or whether the amount of energy penetrating the skin gave a too high dose, is unclear. We need further research on initiation of irradiation and influence of irradiation mode to answer those questions.

Conclusions

In clinical practice, the different skin penetration profiles for superpulsed and continuous lasers will have some clinical implications. In addition to different optimal doses as reflected in WALT guidelines, the penetration profile influences skin temperature during LLLT treatment. We found lower thermal effects in dark skin from 904 nm superpulsed laser than from 810 nm continuous laser in one of our earlier studys.[28] This difference in thermal effects from these two lasers can be explained by skin penetration profile. The percentage of energy absorbed in skin during processing time is decreased for superpulsed lasers, whereas it is constant for continuous lasers. In addition, 904 nm superpulsed lasers have better skin penetration initially than do 810 nm continuous lasers (Fig. 2).

Other critical features with the lasers, which are outside the scope of this study, are to what degree output power (peak value), power density (mW/cm^2), and spot size (cm^2) influence skin penetration.

Other important questions, are "What happens to the skin during LLLT irradiation?" and "Are results from lasers' rat skin penetration applicable to humans?" Few LLLT-studies are concerned with sequelae in the skin during irradiation aiming at tissues beneath the skin. This might be because of the absence of side effects such as skin damage or ablation from LLLT treatment.[3] To the latter issue, the photoacceptor processes from irradiation by near infrared light are believed to be the same in all mammalian cells, catalyzed by cytochrome c oxidase.[29] Stratum corneum and skin thickness in both rats[30] and humans[31] differs with body site. Most irradiation of the skin is absorbed or scattered in stratum corneum of epidermis,[32] and there is considerable similarity in this skin layer thickness between rats[30] and humans.[31] On the other hand, within the research areas of photodynamic therapy and drug metabolisms, the degree of skin permeability and percutaneous absorption differs between rats and humans.[33–36] Further histology investigations after single and repeated LLLT irradiation of rat and human skin flaps are recommended to elucidate if the observed changes over time in 904 nm penetration are irreversible or permanent.

Author Disclosure Statement

No competing financial interests exist.

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Paper III

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ORIGINAL ARTICLE

An experimental study of low-level laser therapy in rat Achilles tendon injury

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Abstract The aim of this controlled animal study was to investigate the effect of low-level laser therapy (LLLT) administered 30 min after injury to the Achilles tendon. The study animals comprised 16 Sprague Dawley male rats divided in two groups. The right Achilles tendons were injured by blunt trauma using a mini guillotine, and were treated with LLLT or placebo LLLT 30 min later. The injury and LLLT procedures were then repeated 15 hours later on the same tendon. One group received active LLLT (λ= 904 nm, 60 mW mean output power, 0.158 W/cm^2 for 50 s, energy 3 J) and the other group received placebo LLLT 23 hours after LLLT. Ultrasonographic images were taken to measure the thickness of the right and left Achilles tendons. Animals were then killed, and all Achilles tendons were tested for ultimate tensile strength (UTS). All analyses were performed by blinded observers. There was a significant increase in tendon thickness in the active LLLT group when compared with the placebo group ($p<0.05$) and there were no significant differences between the placebo and uninjured left tendons. There were no significant differences in UTS between laser-treated, placebo-treated and uninjured tendons. Laser irradiation of the Achilles tendon at 0.158 W/cm^2 for 50 s (3 J) administered within the first 30 min after blunt trauma, and repeated after 15 h, appears to lead to edema of the tendon measured 23 hours after LLLT. The guillotine blunt trauma model seems suitable for inflicting tendon injury and measuring the effects of treatment on edema by ultrasonography and UTS. More studies are needed to further refine this model.

Keywords LLLT · Acute injury · Rat Achilles · Ultrasonographic imaging · Edema · Ultimate tensile strength

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Introduction

Animal models are commonly used in tendon disorder research. They have the advantages of incorporating invasive evaluation techniques, and the possibility for detailed tissue examination and analysis of biochemical substances. These models may be useful in reproducing some aspects of human tendon disorders because in animal models it is easier to control single factors.

In experimental studies on animal tendons, partial or total surgical tenotomy is the most commonly used method for inducing injury [1–8]. Another, nonsurgical, method of injury induction has been introduced in a few studies of tendons in small rodents [9–11]. In this model, an external blunt trauma is inflicted by a mini guillotine where a block falls down from a fixed height and crushes the tendon. After such an injury in a mini guillotine, significant histological changes have been found when compared to healthy tendons [9–11]. However, other relevant outcome measures such as edema and the tendon's ability to withstand load, have not yet been investigated.

The primary purpose of tendons is to transmit tensile load from muscles to bone insertions. Their biomechanical properties can be measured by their ultimate tensile strength (UTS). UTS is an outcome measure of a the tendon's ability to tolerate tensile load and elongation [12]. In two studies on rat tendons where UTS was used as an outcome measure, healthy rat Achilles tendon had UTS values of 42.5±5.5 N (mean±SD) [13] and 48±11.0 N [14].

Low-level laser therapy (LLLT) is considered to act in a biomodulatory manner through light absorption by photoreceptors, which stimulates cells and modulates inflammatory processes [15–19]. Studies performed in a variety of different pathological conditions including injured animal tendons are frequent in the LLLT literature. In most of these LLLT studies, tendons were treated daily for 3 to 21 days [1–5, 9, 11]. Observed histopathological changes in tendons receiving LLLT include increased collagen production [1], improved collagen bundle organization [2–4, 11], and an increased number of small blood vessels [9]. Some studies have investigated the effect of LLLT within the first 24 h after an acute inflammation. After induction of inflammation followed by three or four LLLT sessions, tissue receiving LLLT exhibited reduced concentrations of inflammatory markers and cells compared to no-treatment controls [20, 21].

In the clinical setting tendon disorders are common. Tendon loading seems to be a risk factor for developing tendon disorders, particularly in the upper extremities among manual workers [22] and in the lower extremities among athletes [23]. Acute tendinitis may occur after unfamiliar repetitive movement, friction and pressure during tendon loading, and is often seen in the Achilles tendons of military recruits after long marches [24]. Chronic tendinopathies have a more complex etiology and manifestation, and development of chronic tendinopathies may be gradual and less clearly associated with tendon loading episodes. Age seems to be of importance, and partial or complete tendon ruptures are strongly correlated with age [25]. Developing experimental animal models which accurately mimic the clinical situation in chronic tendinopathies is a challenge, and the ultimate experimental model has yet to be found [26]. Soslowsky et al. [27] developed a rat model of repetitive loading of the supraspinatus in treadmill running, but some aspects including genetics and the influence of pre-experimental structural tendon defects, have not been catered for. Another, as yet unused, possibility for mimicking tendinopathies is to repeat infliction of injury by blunt trauma.

During the last two decades, ultrasonographic imaging (US) has become a popular diagnostic tool for assessment of musculoskeletal disorders [28]. US is reported to have high accuracy in diagnosing disorders in superficial human tendons [28–30].

The aim of this study was to investigate the effects of LLLT administered 30 min after an Achilles tendon injury inflicted by a mini guillotine. The outcome was measured as tendon edema on US and tendon tensile strength.

Method

Animals

The study animals comprised 16 male rats (Sprague Dawley SD M; Taconic Europe, Denmark) weighing 250–300 g, which were divided in two groups. The rats were housed four and four in individually ventilated cages under a light cycle of 12 hours light/12 hours dark, in an atmosphere of humidity 55% and a temperature range of 20–22°C, and with water and food ad libitum.

Instruments

Mini guillotine The mini guillotine comprised a block weight of 200 g with a blunt edge 2 mm wide that was dropped from 20 cm guided by supports (Fig. 1).

Laser The laser emitted radiation at a wavelength 904 nm (Irradia, Sweden) and was operated in pulsed mode. The peak power was 20 W, the pulse width 200 ns and the frequency 700 Hz. The mean output power was 60 mW, the spot size 0.38 cm^2 and the power density 60 mW/0.38 cm^2 (=0.158 W/cm^2). Two identical single diode laser probes were use, one with active laser and the other an inactive/placebo probe. Irradiation was applied for 50 s. Thus 3 J was delivered from the active laser.

Ultrasonography The US instrument was a GE Logiq e (GE Healthcare, Minneapolis, MN) with a 12 MHz linear probe.

Material test system A servohydraulic testing machine (MTS 810; MTS, Minneapolis, MN) equipped with a calibrated load cell of 500 N and a position transducer of 100 mm was used.

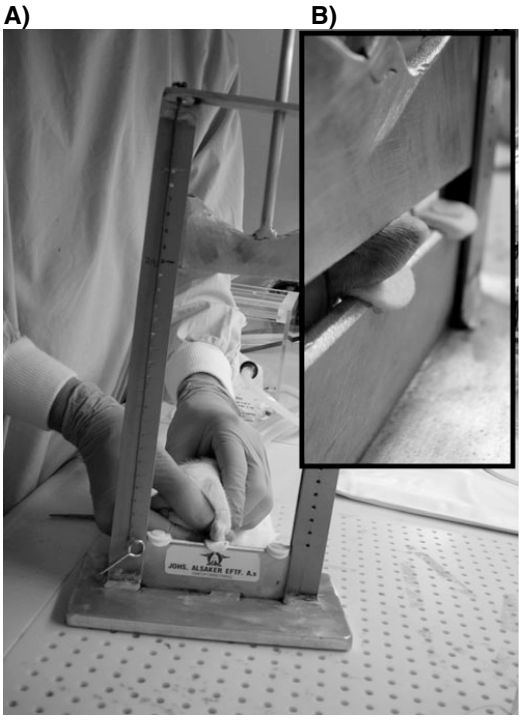

Fig. 1 **a** Mini guillotine with rat positioned for Achilles injury. **b** (*insert*) Close-up of the tendon crush location

The deformation rate was 0.25 mm/s. The load and deformation data were sampled continuously during the test.

Experimental procedure

One investigator (J.M.B.) labeled one laser probe with blue tape and the other with yellow tape. The code for active and placebo probes were not disclosed until the statistical analysis was done. The laser probe output was then measured to determine which probe had been the active one. The main outcome measures were tendon thickness (including the peritendon) as measured on ultrasonographic images and UTS. The study was approved by the local animal laboratory committee at the University of Bergen (application no. 20102676).

The experimental procedure (Fig. 2) was carried out in six steps:

1. The animal was anesthetized with isoflurane (Isoba) using an Isotec vaporizer. Under anesthesia, the animal's right ankle was positioned in full dorsal flexion in the mini guillotine such that the edge of the block hit just proximal to the insertion on the calcaneus (Fig. 1). This procedure was carried out by J.J. After injury, the animal's tail was marked to distinguish it from others in the cage, and was put back into the cage. All animals in each group were subjected to this procedure.
2. Half an hour after injury, the animal was again anesthetized and the injured area was treated for 50 s with either active (3 J) or placebo LLLT delivered to one single point (performed by J.J.). A new mark was put on the tail. This treatment procedure was repeated in all eight animals in each group.
3. The next day, 15 h after the first injury, the same area of the right Achilles tendon was again injured using the guillotine according to the procedure in step 1 and half an hour after later the area was treated for 50 s with LLLT according to the procedure in step 2. This procedure was performed by J.J.
4. On day three, 23 h after the second injury, US was performed bilaterally on the right and left Achilles tendon (carried out by S.H.). The imaging depth was set to 2 cm, with three focus areas around the first centimeter (Fig. 3). The animal was anesthetized and the Achilles tendon area was scanned in both the longitudinal and transverse planes, two images in both planes. The longitudinal plane was scanned with the whole length of the Achilles tendon in the image. Tendon thickness (including peritendon) in the longitudinal plane images was measured from the os calcaneus up to the deeper layer of the skin (Fig. 3). When scanning the Achilles tendon and the adjacent structures in the transverse plane the probe was moved distally from the knee until it met the os Calcaneus, and images were acquired in this position. The thickness in the transverse plane was measured as the vertical distance within the anterior and posterior border of the peritendon. In the statistical analyses the averages of the measurements from two US images were used. Each animal's tail was marked after the US scan was complete.
5. The animals were anesthetized 2 h later and given 0.4 ml intraperitoneal anesthetic (Mebumal 50 mg/ml; 1 ml contains 54.9 mg pentobarbital sodium, 400 g propylene glycol, 150 mg strong alcohol and distilled water to 1 ml). The animals were killed by injection of 1 ml saturated potassium chloride solution to cause cardiac arrest. The skin overlying the right gastrocnemius muscle was released from the calcaneus to the proximal part of the triceps surae muscle. The Achilles tendon was then released with scissors, and the muscle fibers were removed from the tendons with a scalpel. Water containing 9% sodium chloride was sprayed over the tendon. The tendon was put into a small plastic bag, labeled and stored in a refrigerator at 4°C. The same

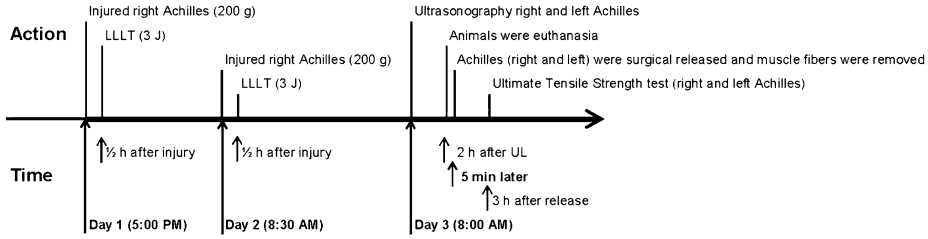

Fig. 2 The experiment time schedule

procedure was used for the left Achilles, and on all animals. This was carried out by J.J.

6. The tendons were subjected to UTS testing 3 h after release. The proximal end of the Achilles tendon was sprinkled with 250 μm alumina powder before it was gripped in a clamp to prevent sliding. A special conically designed grip for the distal end of Achilles tendon was used. In this grip the os calcaneus was used as a plug to obtain the optimal tendon grip [31, 32] (Fig. 4). The maximal load for each tendon at failure was recorded. The procedure was performed by OJ.L. and N.R.G.

Statistical analysis

Differences between injured right Achilles and healthy left Achilles tendons were analyzed using a paired *t*-test, and differences between groups were analyzed using the *t*-test in SPSS (version 18) from Microsoft.

Results

The thickness of the injured right and the healthy left Achilles tendons in the longitudinal US images were significantly different ($p<0.05$) in the active LLLT group (Table 1), but were not significantly different in the placebo LLLT group ($p=0.35$; Table 1). The mean thickness of the injured right Achilles tendon was 0.93±0.03 mm in the active LLLT group and 0.73±0.07 mm in the placebo LLLT group, and the mean thickness of the healthy left Achilles tendon (including the peritendon) was 0.69±0.07 mm and 0.70±0.10 mm, respectively.

In the transverse US images the difference between the injured right and the healthy left Achilles tendon was

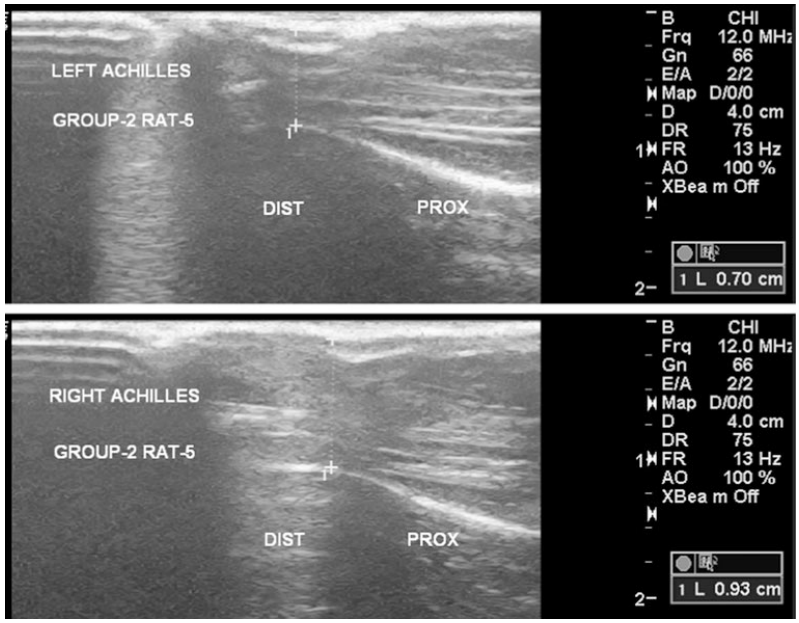

Fig. 3 Longitudinal US images with tendon thickness measurements. Active LLLT group 2) left and right Achilles tendon (rat 5), 0.70 mm and 0.93 mm, respectively

Fig. 4 A rat Achilles tendon at the moment of rupture during UTS testing

significant ($p<0.05$) in the active LLLT group (Table 1), but was not significant the placebo LLLT group ($p=0.16$; Table 1). The mean transverse thickness of the injured right Achilles tendon was 0.70±0.10 mm in the active LLLT group and 0.63±0.14 mm in the placebo LLLT group. The mean thickness of the healthy left Achilles tendon in the two groups was 0.51±0.07 mm and 0.57±0.07 mm, respectively.

There were no significant differences in UTS between the injured right and healthy left Achilles tendon in the active LLLT group or in the placebo LLLT group (Table 1). The mean UTS in the injured right Achilles tendon was 51.11±9.77 N in the active LLLT group and 57.64±7.80 N in the placebo LLLT group. The mean UTS in the healthy left Achilles tendon was 53.94±9.80 N in the active LLLT group and 59.66±11.86 N in the placebo LLLT group.

Discussion

Biomechanical test methods are commonly used in the evaluation of surgically induced tendon injuries [31–33]. The UTS values are fairly low during the first 2 weeks after tenotomy and previous studies have avoided UTS testing in models where tendons are not seriously weakened by sharp trauma. Measuring UTS after tendon injury induced by a mini guillotine has to our knowledge not previously been reported in the literature. After a standardized injury in a mini guillotine, rat tendons show histopathological changes [9–11]. However, we found no differences in the biomechanical properties as revealed by UTS between injured and healthy rat Achilles tendons 1–2 days after injury. Similar UTS values have been found in rat Achilles tendons subjected to carrageenan injection [34].

A major technical challenge in UTS testing of healthy rat Achilles tendons is to obtain a reliable, nonsliding grip of the proximal end of the tendon. We have found only two studies investigating UTS of healthy rat Achilles tendons, possibly because grip slippage before the tendon ruptures. In this study we succeeded in developing a grip with a combination of rolling the tendon around conical anchors, and the application of alumina powder to enhance friction. Thus, grip-induced rupture was avoided, and most tendons ruptured a few millimeters from the distal grip with forces up to 75.2 N.

The standard deviation of the UTS of healthy rat Achilles tendons is ±10 N (present results, and references [14] and [34]) and the difference between healthy and injured tendons is typically 2–3 N. Thus, in future studies with the mini guillotine and UTS, the injury procedure will have to be refined in order to weaken the tendon sufficiently to obtain significant decreases in UTS values after injury.

An irradiation dose of 3 J is commonly used in clinical practice in human inflammatory conditions and was taken from the dose recommendations of the World Association for Laser Therapy (WALT) [35]. In retrospect, this may

Table 1 Thickness (means±SD) of the injured and healthy Achilles tendons and mean differences in thickness as measured by US and UTS ($n=8$)

	Group	View	Tendon thickness (mm)			95% CI	p value
			Right (injured)	Left (healthy)	Difference (injured−healthy)		
US	Placebo LLLT	Longitudinal	0.73±0.03	0.70±0.10	0.03±0.09	−0.04 to 0.11	0.35
		Transverse	0.63±0.14	0.57±0.07	0.07±0.12	−0.03 to 0.17	0.16
	Active LLLT	Longitudinal	0.93±0.03	0.69±0.07	0.24±0.07	0.18 to 0.30	0.00*
		Transverse	0.70±0.10	0.51±0.07	0.19±0.10	0.10 to 0.27	0.00*
UTS	Placebo LLLT		57.64±7.80	59.66±11.86	−2.02±15.09	−14.64 to 10.60	0.72
	Active LLLT		51.11±9.77	53.94±9.80	−2.83±10.76	−11.82 to 6.16	0.48

*$p\leq0.05$.

have been too high for rat Achilles tendon pathology. Optimal irradiation doses from 904-nm lasers in rat muscles seem to be closer to 1 J. In a new study with LLLT, doses of 0.1, 0.3, 1.0 and 3.0 J were used to prevent skeletal muscle fatigue and possible muscle damage. All irradiated groups except the group irradiated at a dose of 3.0 J had significantly lower post-exercise creatine kinase activity than the control group immediately after the contraction tests [36]. If these doses translate to rat Achilles tendons, it could explain the nonsignificant differences, but not the increased edema observed in the active LLLT group.

In the current study there was an interval of 15 h between the two LLLT irradiations. There are very few studies in which there was an interval of less than 24 h between irradiations. Thus, we cannot rule out the possibility that an interval of 15 h was too short and resulted in negative effects of LLLT. This is clearly an area where more research is needed. A more likely explanation for the increased edema in the active LLLT group could be an indirect consequence of symptomatic pain reduction from LLLT leading to increased activity causing more edema [37, 38]. Reduced pain sensation from the injured tissue could promote physical activity, and this may result in a temporary increase in edema at the site of injury during the 23 h between the LLLT irradiation session and the US examination [39].

The timing of LLLT irradiation in the acute phase of inflammation is seldom discussed in the literature, but it can be crucial for achieving positive effects. In some animal studies, LLLT treatment has been found to reduce edema, and is typically been measured 4 hours after injury [40–42]. Thus, we did not expect to find an increase in edema after a single LLLT treatment 30 min after injury. We speculate that the unexpected differences between the results may be explained by differences in study design. Scrutiny of the design of other similar LLLT studies showed that LLLT has mainly been administered later than 30 min after injury [42] and/or that the treatment procedure included more than a single LLLT session [2, 3, 9, 21, 40, 41].

It is known that LLLT energy acts on biological processes [43]. We therefore considered the cascade of responses in the acute phase of inflammation, and in particular which processes are dominant in the first half hour. The immediate response in the innate immunity after an injury is activation of signal transduction mechanisms. Immediate genes activated within the first half hour of inflammation are mainly transcription factors [44, 45]. Among these expressed cytokine gene is mitogen-activated protein kinase (MAPK) [46]. Three well-characterized subfamilies of MAPK are stress-activated protein kinases/c-Jun N-terminal kinase (JNK), p38 mitogen-activated protein kinase (p38) and extracellular regulated protein kinases (ERK) [44, 47, 48]. Peak times for kinases have been established for JNK, p38 and ERK in tendon cells, smooth muscle cells, endothelial cells and cardiac fibroblasts. JNK, p38 and ERK increase rapidly, and they reach a maximum within 10 to 30 min [49–52]. The impact of JNK, p38 and ERK on edema has been investigated in rodent paw models and brain injury models using inhibitors for JNK, p38 and ERK, respectively. Tested selectively, all these inhibitors reduce the development of edema, and consequently JNK, p38 and ERK may increase edema development [53–56].

The effect from LLLT (632.8 nm, 4.5 mW) on JNK, p38 and ERK has been investigated in skeletal muscle cells. LLLT does not increase JNK and p38 activation or protein expression, but LLLT induces protein translation through ERK pathways [57, 58]. Perhaps the unexpected increase in edema development in the LLLT group in our study can be explained through a stimulation of the ERK pathway. But this is speculative as we did not measure any of the outcomes needed to confirm or refute this theory.

We also explored studies with LLLT irradiation within the first half hour after injury (injection or surgery) with regard to edema development. In a previous study by members of our research group [40], different timing protocols for LLLT (650 nm) were used. Carrageenan was injected into rat paws and edema was measured 1 to 4 h later. The main conclusion was that LLLT has an antiinflammatory effect in most cases, but one group treated with LLLT had developed increased edema 10 min after injection compared to controls.

Some clinical studies in dentistry have evaluated the effect of LLLT on edema development immediately after surgery [59–64]. The timing of LLLT administration was not explicitly reported, but in two studies report the duration of surgery was 19 min [63, 64]. In one study, LLLT given immediately after surgery was found to have increased edema at 3 days, but not at 7 days [62]. Two studies with adequate doses found reduced edema [63, 64]. In three studies LLLT doses did not meet the WALT dose recommendations for LLLT. One study used a scanning mode for application of the laser beam [61], and the other two used doses that were too low (<1 J) [59, 60].

Even though the results regarding edema development in studies in which LLLT was used within the first half hour after injury are arbitrary, it is not unlikely that LLLT irradiation within the first half hour after an injury will temporarily increase edema. This finding has important clinical implications, as it has been an open question as to when is the earliest time-point for effective LLLT administration. Interestingly, in some studies LLLT has been administered before muscle damage or the onset of inflammatory process with good results on other outcome measures [36, 65]. This indicates that the negative effect on edema development is restricted to inflamed tissue and the first half hour after the onset of inflammation.

Our decision to use the mini guillotine model was an attempt to mimic a tendon disorder with inflammatory components. Signs of inflammation are present in acute and mild tendinopathies [66, 67]. In previous studies using the mini guillotine injury method in rodents, the injured tendons have shown inflammatory reactions [9–11]. The significant increase in thickness of the injured tendons supports the suitability of the mini guillotine model for causing Achilles tendinopathy in the rat. The mini guillotine model has the advantage of being easier to use than overuse models such as the treadmill running model of Soslowsky et al. [68]. None of these models involves penetration of the skin and therefore they avoid the risk of infection during the injury procedure. The surgical procedure of tenotomy does introduce a risk for infection, and it may also be questioned as to how relevant this model for investigation of tendinopathies.

The use of animal models in research on human tendinopathies has its limitations. There are obvious anatomical differences between bipeds and quadrupeds. This affects the validity of using animal models to study tendinopathies, and limits extrapolation of the findings in animal studies to human tendinopathies. Whether effective laser parameters in small rodent models can be extrapolated to humans remains uncertain and additional clinical studies in humans to verify or repute the findings are called for.

Conclusion

Laser irradiation at 0.158 W/cm^2 for 50 s (3 J) administered within the first half hour after two blunt trauma injuries15 h apart appeared to increase edema in the rat Achilles tendon measured at 23 h after LLLT.

The UTS of the rat Achilles tendon was not decreased significantly after two blunt traumas inflicted by a mini guillotine. This experimental model appears useful for investigating the optimal timing and effect of therapies in acute tendon injuries. In this model, edema can be reliably measured by US, and tendon UTS can be reliably tested in both healthy and weakened rat Achilles tendons. There is a need to further refine the experimental procedure to achieve a weakening of the rat Achilles tendon. Further studies with this model should include variations in the intervals between traumas, the timing of treatment, the number of LLLT sessions and the power densities.

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